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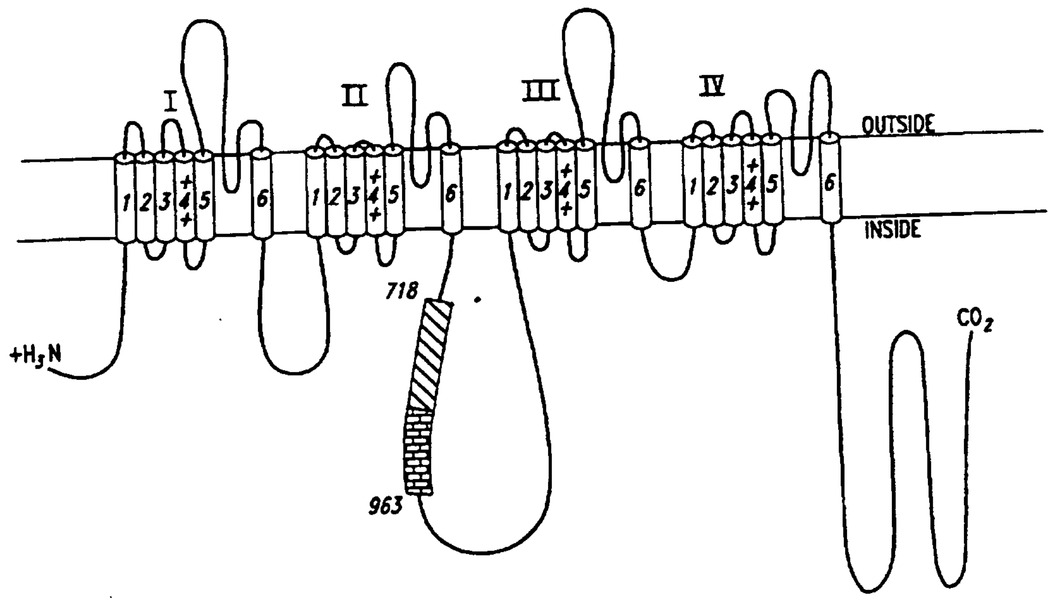

(57) Abstract

Methods and compositions related to the identification of compounds that block neurotransmitter release are disclosed. Using the methods of the present invention, candidate compounds may be screened for the ability to bind to presynaptic calcium channels such that the docking of presynaptic vesicles to presynaptic calcium channels will be inhibited. The present invention also discloses peptides useful in the screening methods.

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Description

METHODS AND COMPOSITIONS FOR SCREENING FOR PRESYNAPTIC CALCIUM CHANNEL BLOCKERS

Technical Field

The present invention is generally directed toward assays and compositions for identifying compounds that block neurotransmitter release. This invention is more particularly related to screening candidate compounds for the ability to block presynaptic calcium channels.

Background of the Invention

The release of neurotransmitters from presynaptic terminals is the final response of a nerve to the excitatory and inhibitory inputs that converge upon it. Neurotransmitter release at the presynaptic terminal of neurons is primarily initiated by the entry of calcium through voltage-gated calcium channels (Smith and Augustine, *Trends Neurosci. 11*:458-464, 1988; Robitaille et al., *Neuron 5*:773-779, 1990). Exocytosis of synaptic vesicles occurs at specialized regions of the nerve terminal called active zones. These zones may contain clusters of presynaptic calcium channels that supply calcium for neurotransmitter release (Pumplin et al., *Proc. Natl. Acad. Sci. USA 78*:7210-7214, 1981; Pumplin, *J. Neurocytol. 12*:317-323, 1983; Zucker, *J. Physiol. 87*:25-36, 1993). The entry of calcium through voltage-gated calcium channels couples electrical activity to secretion of synaptic vesicles. Synaptic transmission is initiated within 200 μs after the arrival of the action potential at the synaptic terminal. The brief rise in Ca^{++} concentration to the level necessary for exocytosis likely occurs only in proximity to the calcium channels (Llinás et al., *Biophys. J. 33*:289-322; Cope and Mendell, *J. Neurosci. 47*:469-478, 1982).

A combination of electrophysiological and pharmacological criteria have defined four main types of high-voltage-activated calcium channels that are widely distributed in mammalian neurons. These are ω-conotoxin-GVIA-sensitive N-type calcium channels, ω-agatoxin IVA-sensitive and ω-conotoxin-MVIIC-sensitive P-type and Q-type calcium channels, and dihydropyridine-sensitive L-type calcium channels (for reviews see Bean, *Annu. Rev. Physiol. 51*:367-384, 1989; Hess, *Ann. Rev. Neurosci. 13*:337-356, 1990;

Tsien et al., *Trends Pharmac. Sci. 12*:349-354, 1991; Miller, *J. Biol. Chem. 267*:1403-1406, 1992; Zhang et al., *Neuropharmacology 32*:1075-1088, 1993). Several lines of evidence indicate that N-type channels, at least in part, are responsible for the calcium influx that triggers transmitter release in many neurons. Antibodies against ω-conotoxin GVIA (ω-CTx GVIA) or fluorescent toxin derivatives label active zones on the terminals of motor neurons at the frog neuromuscular junction (Robitaille et al., *Neuron 5*:773-779, 1990; Cohen et al., *J. Neurosci 1*:1032-1039, 1991). Immunocytochemical studies with specific site-directed anti-peptide antibodies indicate that N-type channels are located along the length of dendrites and in synapses formed on the dendrites of many brain neurons (Westenbroek et al., *Neuron 9*:1099-1115, 1992). In contrast, antibodies to L-type channels recognize calcium channels in cell bodies and proximal dendrites, but give no detectable staining of presynaptic terminals in brain (Ahlijanian et al., *Neuron 4*:819-832, 1990). In addition, ω-CTx-GVIA inhibits transmitter release in a variety of mammalian neuronal preparations (Hirning et al., *Science 239*:57-60, 1988; Horne and Kemp, *Br. J. Pharmacol. 103*:1733-1739, 1991; Takahashi and Momiyama, *Nature 366*:156-158, 1993; Luebke et al., *Neuron 11*:895-902, 1993; Turner et al., *Proc. Natl Acad Sci USA 90*:9518-9522, 1993; Wheeler et al., *Science 264*:107-111, 1994), thus supporting the hypothesis that N-type channels play a role in controlling neurotransmitter release in the central nervous system. Similarly, P-type and Q-type channels (collectively P/Q-type channels) have been implicated in neurotransmitter release in mammalian neurons. N-type channels appear to be the dominant form in presynaptic terminals of the peripheral nervous system, and P/Q-type channels in presynaptic terminals of the central nervous system.

Molecular cloning has identified the primary structures of the main pore-forming $\alpha 1$ subunit of five distinct classes of calcium channels (classes A, B, C, D, and E) found in rat brain. Cloned neuronal $\alpha 1_C$ and $\alpha 1_D$ subunits are components of L-type channels, while the $\alpha 1_B$ subunit is a component of N-type channels (Dubel et al., *Proc. Natl. Acad. Sci. USA 89*:5058-5062, 1992; Williams et al., *Neuron 8*:71-84, 1992a; Williams et al., *Science 257*:389-395, 1992b; Westenbroek et al., *Neuron 9*:1099-1115, 1992; Stea et al., *Neuropharmacology 32*:1103-1116, 1993). $\alpha 1_A$ encodes Q-type calcium channels and may also encode P-type calcium channels (Snutch and Reiner, *Curr. Opin. Neurobiol. 2*:247-253, 1992; Tsien et al., *Trends Pharmac. Sci. 12*:349-354, 1991; Mori et al., *Nature 350*:398-402, 1991; Sather et al., *Neuron 11*:291-303, 1993; Zhang et al., *Neuropharmacology 32*:1075-1088, 1993). The deduced amino acid

sequence of $\alpha 1_B$ shares overall structural features with other calcium channel $\alpha 1$ subunits. It is composed of four predominantly hydrophobic homologous domains (I-IV) that are linked by intracellular hydrophilic loops of various lengths.

The traditional approach to blocking neurotransmitter release has been to use compounds that bind to the neuronal voltage-gated calcium channels in a manner such that calcium entry through the channels is blocked. One of the difficulties in such an approach is the lack of specificity. As noted above, voltage-activated calcium channels that are found at sites in the body other than at presynaptic terminals appear to share structural features responsible for the movement of calcium through the channels. Accordingly, compounds that interact with the pore portion of calcium channels to block calcium entry into presynaptic nerve terminals will also block calcium channels at other sites throughout the body. Therefore, the traditional compounds for blocking neurotransmitter release have undesired side effects due to the blockade of additional calcium channels.

Due to the limited success for previously suggested compounds for the inhibition of neurotransmitter release, there is a need in the art for methods and compositions to screen for new inhibitors with specificity for presynaptic voltage-gated calcium channels. The present invention fulfills this need. and further provides other related advantages.

Summary of the Invention

Current compounds in the art for blocking neurotransmitter release act by inhibiting the calcium influx through calcium channels that triggers transmitter release. As noted above, this approach suffers from problems associated with the inhibition of calcium channels at sites other than presynaptic terminals of neurons. An advantage of the methods and compositions of the present invention is that compounds are screened for the ability to inhibit the docking of presynaptic vesicles to presynaptic calcium channels, rather than for the ability to inhibit calcium influx through the channels.

Briefly stated, the present invention provides a variety of methods and compositions related to screening compounds for the ability to inhibit the interaction between presynaptic calcium channels and presynaptic vesicles (*e.g.*, by a compound's ability to bind to a selected presynaptic calcium channel-like peptide). In one aspect, the present invention provides methods of screening for

compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles. In one embodiment, the method comprises the steps of: (a) contacting a calcium channel-like peptide with a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound, wherein the peptide is able to bind syntaxin or SNAP-25; and (b) detecting the presence or absence of binding between the peptide and the candidate compound, thereby determining whether the candidate compound bound to the peptide.

In another embodiment of the method, step (a) further includes syntaxin or a syntaxin-like peptide under conditions sufficient to permit binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide; and step (b) comprises detecting the presence or absence of binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide, thereby determining whether the candidate compound inhibited the binding.

In another embodiment of the method, step (b) comprises the steps of: (b) adding syntaxin or a syntaxin-like peptide to the reaction mixture of the candidate compound and the calcium channel-like peptide of step (a) under conditions sufficient to permit binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide, and (c) detecting the presence or absence of binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide, thereby determining whether the candidate compound inhibited the binding.

In another embodiment of the method, step (a) further includes SNAP-25 or a SNAP-25-like peptide under conditions sufficient to permit binding between the calcium channel-like peptide and the SNAP-25 or the SNAP-25-like peptide; and step (b) comprises detecting the presence or absence of binding between the calcium channel-like peptide and the SNAP-25 or the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

In another embodiment of the method, step (b) comprises the steps of: (b) adding SNAP-25 or a SNAP-25-like peptide to the reaction mixture of the candidate compound and the calcium channel-like peptide of step (a) under conditions sufficient to permit binding between the calcium channel-like peptide and the SNAP-25 or the SNAP-25-like peptide, and (c) detecting the presence or absence of binding between the calcium channel-like peptide and the SNAP-25

or the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

In any of the above embodiments, the calcium channel-like peptide may be such that it has at least 87 amino acid residues selected from amino acid 717 to amino acid 1143 of an α_1 subunit of a N-type or Q-type calcium channel. The 87 or more amino acids are sequential.

In a related aspect, the present invention provides peptides derived from, or based upon, a selected portion of a presynaptic calcium channel amino acid sequence. In one embodiment, the peptide consists essentially of the amino acid sequence of Figure 11A from alanine, amino acid 773, to aspartic acid, amino acid 859. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to aspartic acid, amino acid 859. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to arginine, amino acid 963. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to cysteine, amino acid 1141. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11A from an amino acid positioned between glutamic acid, amino acid 718, and alanine, amino acid 773, to an amino acid positioned between aspartic acid, amino acid 859, and cysteine, amino acid 1141. In another embodiment, the peptide consists essentially of an amino acid sequence of between 246 to 424 amino acid residues in length, wherein the amino acid sequence contains an amino acid sequence having at least 60% sequence similarity with the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to arginine, amino acid 963.

In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11B from alanine, amino acid 772, to aspartic acid, amino acid 858. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to aspartic acid, amino acid 858. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to threonine, amino acid 1036. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to cysteine, amino acid 1143. In another embodiment, the peptide consists essentially of the amino acid sequence of Figure 11B from an amino acid positioned between glutamic acid, amino acid 717, and alanine, amino acid 772, to an amino acid positioned between aspartic acid, amino acid

858, and cysteine, amino acid 1143. In another embodiment, the peptide consists essentially of an amino acid sequence of between 246 to 427 amino acid residues in length, wherein the amino acid sequence contains an amino acid sequence having at least 60% sequence similarity with the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to arginine, amino acid 962.

In yet another embodiment, the peptide consists essentially of the amino acid sequence of hBI of Figure 15 from glutamic acid, amino acid 722, to arginine, amino acid 1036.

In another aspect, a syntaxin-like peptide is provided and consists essentially of the amino acid sequence of Figure 12 from isoleucine, amino acid 181, to glycine, amino acid 288.

In another related aspect of the present invention, the particular peptides described above are utilized in methods of screening for compounds that inhibit the interaction between presynaptic calcium channel and presynaptic vesicles. In one embodiment, the method comprises the step of: (a) contacting a presynaptic calcium channel-like peptide as described above with a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound, and (b) detecting the presence or absence of binding between the peptide and the candidate compound. thereby determining whether the candidate compound bound to the peptide.

In another embodiment, the method comprises the steps of: (a) incubating a candidate compound, a presynaptic calcium channel-like first peptide as described above. and syntaxin or a syntaxin-like second peptide under conditions sufficient to permit binding between the first peptide and syntaxin or between the first peptide and the second peptide. and (b) detecting the presence or absence of binding between the first peptide and syntaxin or between the first peptide and the second peptide, thereby determining whether the candidate compound inhibited the binding.

In another embodiment, the method comprises the steps of: (a) incubating a presynaptic calcium channel-like first peptide as described above and a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound, to form a reaction mixture, (b) contacting syntaxin or a syntaxin-like second peptide with the reaction mixture under conditions sufficient to permit binding between the first peptide and syntaxin or between the first peptide and the second peptide, and (c) detecting the presence or absence of binding between the first peptide and

syntaxin or between the first peptide and the second peptide, thereby determining whether the candidate compound inhibited the binding.

In another embodiment, the method comprises the steps of: (a) incubating a candidate compound, a presynaptic calcium channel-like first peptide as described above, and SNAP-25 or a SNAP-25-like peptide under conditions sufficient to permit binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide, and (b) detecting the presence or absence of binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

In yet another embodiment, the method comprises the steps of: (a) incubating a presynaptic calcium channel-like first peptide as described above and a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound, to form a reaction mixture, (b) contacting SNAP-25 or a SNAP-25-like peptide with the reaction mixture under conditions sufficient to permit binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide, and (c) detecting the presence or absence of binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 is a drawing depicting the predicted topological structure of the $\alpha 1$ subunit of class B N-type calcium channels. The location of the recombinant His-fusion proteins of calcium channel cytoplasmic domains generated for binding studies are depicted. The filled-in rectangle indicates a region of interaction with syntaxin 1A.

Figure 2 is immunoblots (Panels A and B) that demonstrate the interaction of the cytoplasmic loop $L_{II\text{-}III}$ (718-1145) of $\alpha 1_B$ with syntaxin 1A. Approximately 2 μg of GST-syntaxin (GST-syn) or GST coupled to glutathione-Sepharose 4B beads were incubated with 5-10 μg of the indicated various His-fusion proteins. Specifically-bound proteins were eluted by 15 mM glutathione/50 mM Tris-HCl, pH 8, separated by SDS/PAGE, electrophoretically

transferred to nitrocellulose, and probed for the presence of His-fusion proteins by immunoblotting with anti-T7-Tag antibody. Left lane, aliquots of lysates containing His-fusion proteins as indicated; center lane, eluate from GST-syntaxin affinity matrix; right lane, eluate from GST affinity matrix.

Figure 3 is a schematic representation of the His-fusion proteins containing various sequences from the cytoplasmic loop L_{II-III} (718-1145).

Figure 4 is an immunoblot analysis of the interaction of fusion proteins containing sequences from loop L_{II-III} (718-1145) with syntaxin 1A. The indicated His-fusion proteins were incubated with GST-syntaxin (GST-syn) and GST affinity matrices. Bound proteins were eluted and detected on immunoblots with anti-T7-Tag antibody. To normalize for the amounts of GST-syn or GST fusion proteins bound to affinity matrix and eluted, the blots were stripped and probed with anti-GST antibody.

Figure 5 is a Coomassie blue-stained polyacrylamide gel showing the specificity of the binding of GST-syntaxin to L_{II-III}(718-859). Total bacterial lysate containing His-L_{II-III}(718-859) fusion protein was incubated with glutathione-Sepharose-4B beads prebound by bacterial lysate containing GST-syntaxin fusion protein (GST-syn) or bacterial lysate without any fusion protein (control) as indicated. Complexes of GST-syntaxin and L_{II-III}(718-859) were eluted and analyzed by SDS-PAGE and Coomassie blue staining. Migration positions of GST-syn, L_{II-III}(718-859), and a non-specific glutathione-Sepharose binding bacterial protein of ~70 kDa (*) are indicated.

Figure 6 shows a sequence analysis of the syntaxin-binding site and location of two overlapping helix-loop-helix motifs within the L_{II-III}(773-859) region. Underneath the locations of amino acids used to generate six new His-fusion proteins are shown. The binding activity of each deleted fusion protein is listed in the right column.

Figure 7 is an immunoblot demonstrating that deletion mutants of the helix-loop-helix structure completely abolished syntaxin-binding activity. Approximately 5 μg of His-fusion proteins from four of the six deletion mutants shown in Figure 6 and the complete 87-residue peptide (773-859) were incubated with affinity matrix containing 2 μg of GST-syn or GST. The bound His-fusion proteins were eluted and probed with anti-T7-Tag antibody (top). Controls for quantity and quality of His-fusion proteins are shown in the top right panel. The amounts of GST-syntaxin (GST-syn) or GST fusion proteins attached and eluted from matrix were determined by a second blotting with anti-GST antibody shown in bottom panel.

Figure 8 is an immunoblot showing the interaction of the corresponding regions from $\alpha 1_A$ and $\alpha 1_S$ with syntaxin 1A. His-fusion proteins with the corresponding region (723-868) of the cytoplasmic loop L_{II-III} of class A calcium channel and the entire L_{II-III} loop (670-800) of L-type rabbit skeletal muscle calcium channel (class S) were expressed. The binding assays were performed as described for Figure 2. Aliquots of lysates as indicated were incubated with GST-syntaxin (GST-syn) or GST-glutathione-Sepharose beads. Bound proteins were eluted and resolved by electrophoresis in SDS/PAGE and sequentially probed with anti-T7-Tag (top panel) and anti-GST (bottom panel) antibodies.

Figure 9 is an immunoblot demonstrating the interaction of the amino and carboxyl terminal domains of syntaxin 1A with N-type calcium channels. Two GST-syntaxin fusion proteins containing the amino-terminal sequence, GST-syn-NT(2-190), and the carboxyl-terminal sequence, GST-syn-CT(181-288), were generated and used for affinity matrices. Approximately 2 μg of both His-fusion proteins, L_{II-III}(718-859) and L_{II-III}(773-859), containing the syntaxin-binding site, were loaded on affinity matrices with GST-syn. GST-syn-NT, GST-syn-CT, and GST. As indicated, bound His-fusion proteins were co-eluted with GST-fusion proteins and immunoblotted sequentially with anti-T7-Tag (top panel) and anti-GST (bottom panel) antibodies.

Figure 10 contains graphs demonstrating the inhibition of N-type calcium channel binding to syntaxin 1A by the 87 amino acid binding peptide. The graph in panel A shows the extent of immunoprecipitation of $\alpha 1_B$ and $\alpha 1_B$-syntaxin complex. The class B N-type calcium channels in synaptic membranes were solubilized with digitonin and partially purified by wheat germ agglutinin (WGA)-Sepharose affinity chromatography. The calcium channels were labeled with 500 fmol $[^{125}I]Tyr^{22}$-ω-CTx-GVIA and immunoprecipitated with CNB1 (anti-$\alpha 1_B$), 10H5 (anti-syntaxin). and control mouse IgG as indicated. The immunoprecipitation data are expressed as a percentage of the total labeled channels.

The graph in panel B of Figure 10 shows the direct interaction of ^{125}I-ω-CTx-GVIA receptor and GST-syntaxin. Equal amounts (cpm) of $[^{125}I]$-ω-CTx-GVIA-labeled N-type calcium channels were incubated with affinity matrices containing GST-syntaxin or GST for 3 hr. The beads were washed for three times with PBS, and the amount of bound receptors was assessed by direct counting. The counts from three independent binding data were averaged.

The graph in panel C of Figure 10 displays results of binding competition assays. The binding assays were performed as in panel B except that the competing peptides, His-L_{II-III}(733-859) or His-L_{II-III}(1027-1145) were present. The reduction of binding activity was expressed as a percentage of total binding of labeled receptors to GST-syntaxin in the absence of any competing peptide. The data were averaged from three independent experiments.

Figure 11 shows an alignment of loop L_{II-III}(710-1143) amino acid sequences of rat (Figure 11A) and human (Figure 11B) N-type calcium channels. The sequences have been aligned to maximize the sequence similarity.

Figure 12 depicts the entire amino acid sequence (1-288) of rat syntaxin 1A.

Figure 13 is a drawing depicting the predicted topological structure of the α_1 subunit of the N-type Ca^{++} channel. It is composed of four predominantly hydrophobic homologous domains (I-IV) that are linked by intracellular hydrophilic loops of various lengths. The two syntaxin binding regions in the intracellular loop II are highlighted by shaded boxes.

Figure 14 shows that syntaxin-binding affinity of N-type Ca^{++} channel fusion proteins increases with size. Equal concentrations of GST-syntaxin 1A (5 μM, left lanes) and GST (5 μM, right lanes) were bound to glutathione-Sepharose beads and incubated with various fusion proteins of L_{II-III} of N-type Ca^{++} channels (aa 718-859, 832-963 and 718-963; 10 μM each). The beads were washed and bound proteins were eluted with 15 mM reduced glutathione/50 mM Tris-HCl pH 8. Following SDS-PAGE and electrotransfer to nitrocellulose, the Ca^{++} channel fusion proteins were detected with Anti-T7.Tag monoclonal antibody. Numbers on the right indicate positions of molecular weight markers (in kd). To demonstrate equal concentrations of GST fusion proteins loaded on the beads, Ponceau S staining of the nitrocellulose membrane is shown in the lower panel.

Figure 15 depicts an alignment of the primary structures of the syntaxin-binding segments of the rbA and BI isoforms of α_{1A}. Amino acids are represented by the corresponding one-letter abbreviation, with sequence differences depicted by use of the lower case letter. cDNAs encoding a human BI isoform of the α_{1A} subunit were isolated from a small cell carcinoma line and from human hippocampus and sequenced as described below. The sequences of the rabbit BI isoform of α_{1A} (Mori et al., *Nature 350*:398-402, 1991), the rat rbA isoform of α_{1A} (Starr et al., *Proc. Natl. Acad. Sci. USA 88*:5621-5625, 1991),

and the human hBI isoform of α_{1A} are compared, and the positions of the N- and C-termini of the fusion proteins used in these experiments are noted by arrows.

Figure 16 shows that α_{1B} and the hBI isoform of α_{1A} bind to the C-terminal region of syntaxin 1A. Fusion proteins containing aa 2-190 (N-terminus, lanes 1-3) and aa 181-288 (C-terminus, lanes 4-6) of Syntaxin 1A and GST (lanes 7-9) were bound to glutathione-Sepharose beads. Following a brief wash, beads were incubated with 10 μM of the indicated fusion proteins from α_{1B} (rbB) (A) and hBI (B), respectively. Bound material was fractioned by SDS-PAGE and visualized by immunoblotting.

Figure 17 shows that α_{1A} and α_{1B} bind to the presynaptic protein SNAP-25. GST-SNAP-25 beads (5 μM) were incubated with equal concentrations of various fusion proteins containing L_{II-III} of rbB and the rbA and hBI isoforms of α_{1A} as indicated. Bound material was fractioned by SDS-PAGE and visualized by immunoblotting. Note that the blot is overexposed in order to demonstrate weak binding of both rbA 724-981 and hBI 843-1036. Ponceau S staining of the nitrocellulose membrane is shown below; numbers on the right indicate positions of molecular mass markers (in kDa).

Figure 18 shows that α_{1A} and α_{1B} compete for the same binding region on SNAP-25. GST-SNAP-25 beads (5 μM) were incubated with equal concentrations of hBI 722-1036 and increasing concentrations of rbB 718-963 as indicated. Bound material was fractioned by SDS-PAGE and visualized by immunoblotting as described below. Ponceau S staining of the nitrocellulose membrane is shown in the lower panels.

Detailed Description of the Invention

The identification of calcium channel blockers that specifically inhibit the presynaptic calcium channels involved in release of neurotransmitters, such as glutamate, at excitatory synapses in the central nervous system would be therapeutically beneficial (*e.g.*, in preventing the neuronal cell death that accompanies cerebral ischemia). As described above, compounds that block the calcium conductance activity of these channels are not specific. The present invention provides a screening approach, for compounds which prevent neurotransmitter release triggered by presynaptic calcium channels, that is based on inhibition of the docking of presynaptic vesicles to active zones containing the presynaptic calcium channels.

The disclosure of the present invention shows that presynaptic calcium channels possess a site for binding syntaxin and SNAP-25, proteins anchored in the presynaptic plasma membrane, and that this site has a number of uses, including to screen for compounds that block neurotransmitter release. A compound that inhibits syntaxin or SNAP-25 binding to the site (*e.g.*, by the compound occupying the site) will interfere with the interaction between presynaptic calcium channels and presynaptic vesicles docked to syntaxin or SNAP-25. With the methods and compositions of the present invention, candidate compounds may be screened for those that interrupt the interaction between presynaptic calcium channels and synaptic vesicles by interaction with the herein disclosed target site, which is on the calcium channel but is not involved in calcium influx itself. Thus, the present invention permits the identification of compounds with the desirable properties that, although calcium would still enter through the channel, transmitter release would not occur because the synaptic vesicle would not be properly docked to respond to the locally-increased calcium concentration.

As shown by the disclosure provided herein, N-type presynaptic calcium channels possess amino acid sequences that interact specifically with syntaxin, a presynaptic plasma membrane protein. In particular, syntaxin is shown to directly interact with two adjacent interacting regions within the cytoplasmic loop (L_{II-III}) between homologous repeats (domains) II and III of N-type calcium channels. Loop L_{II-III} is a sequence of about 428 amino acids composed from about residues 718 to about 1145 (Figure 11A) of the $\alpha 1$ subunit of N-type calcium channels. While the entire L_{II-III} sequence may be used, it is not required as portions of the sequence will suffice. For example, an amino-terminal sequence of about 320 amino acids from about residues 718 to 1037 in Figure 11A (or from about residues 717 to 1036 in Figure 11B) interact with syntaxin. Other examples of sequences that interact with syntaxin include an amino-terminal sequence of about 142 amino acids from about residues 718 to 859 in Figure 11A, or an amino-terminal sequence of about 246 amino acids from about residues 718 to 963 in Figure 11A (or from about residues 717 to 962 in Figure 11B). Further, portions of such amino-terminal sequences may be used for interaction with syntaxin. For example, a sequence of about 87 amino acids (from about residues 773 to 859) interacts specifically with syntaxin.

Similarly, while the entire syntaxin protein may be used, it is not required as portions of the sequence will suffice. For example, a carboxyl-terminal sequence of about 108 amino acids (from about residues 181 to 288 in

Figure 12) interacts with L_{II-III} or portions thereof. It will be evident to those of ordinary skill in the art that based on the disclosure provided herein that smaller segments of syntaxin may be identified that retain the characteristic high affinity interaction described herein between syntaxin and N-type calcium channels.

The disclosure of the present invention also shows that the corresponding two adjacent interacting regions within loop L_{II-III} of the $\alpha 1$ subunit of the P/Q-type calcium channels similarly interact directly with syntaxin. In addition, for example, a portion of the L_{II-III} sequence of the P/Q-type from about residue 722 to 1036 (*e.g.*, Figure 15) interacts specifically with syntaxin. Again, the L_{II-III} sequence of the P/Q-type binds to the carboxyl-terminal one-third of syntaxin. The P/Q-type and N-type channels compete for the same binding region on syntaxin.

As also shown by the disclosure provided herein, N-type and P/Q-type calcium channels possess amino acid sequences that interact specifically with another presynaptic plasma membrane protein, SNAP-25 (the synaptosome-associated protein of 25 kD). The same two adjacent regions on the N-type and P/Q-type channels that bind syntaxin are also able to bind to SNAP-25. The affinity for binding SNAP-25 is comparable to the affinity for binding syntaxin. The α subunit of N-type (α_{1B}) and P/Q-type (α_{1A}) channels compete for binding to SNAP-25. Therefore, α_{1A} and α_{1B} interact with identical or overlapping regions on SNAP-25 as well as on syntaxin.

Based on the foregoing, the present invention discloses peptides useful for targeting compounds to disrupt the interaction (*e.g.*, to prevent neurotransmitter release triggered by presynaptic calcium channels) between syntaxin and N-type or P/Q-type calcium channels, or between SNAP-25 and N-type or P/Q-type calcium channels. Peptides may be produced in a variety of ways well known to those in the art. For example, peptides may be derived from native proteins, prepared by synthetic chemistry methodology (including automated peptide synthesis, *e.g.*, using an instrument available from Applied Biosystems, Inc., Foster City, CA), or produced by recombinant DNA techniques (including as fusion proteins expressed in microorganisms such as bacteria). Preferred peptides include those which correspond to the amino acid sequences in Figure 11A from amino acid 718 to 1141, from 718 to 1037, from 718 to 963, from 718 to 859, and from 773 to 859.

Based upon the present disclosure, it will be evident to those in the art that useful peptides may be created which contain a sequence identical, or similar, to that of the amino acids in Figure 11A. For example, the amino

terminus of such a peptide may begin at any of the amino acids positioned between (as used herein "between" includes the recited amino acids) amino acid 718 to 773 and the carboxyl terminus may end at any of the amino acids positioned between amino acid 859 to 1141. Alternatively, for example, a peptide may be similar in length to the peptides of amino acids from 718 to 1141 or 718 to 963 (*i.e.*, have a length of about 246 to 424 amino acid residues) and contain an amino acid sequence having at least 60% sequence similarity with the amino acid sequence of Figure 11A from amino acids 718 to 963. Particularly preferred peptides include those with about 65%, 70%, 75%, 80%, 85% or greater sequence similarity, and those with about 75%, 80%, 85% or greater sequence identity. Sequence similarity is based upon sequence identity plus conservative substitutions of amino acids. Conservative substitutions include interchanges of valine and isoleucine, leucine and isoleucine, aspartic acid and glutamic acid, and others of a similar nature. When such a peptide has more than 246 residues, the additional amino acids may have, but need not have, sequence similarity to the $L_{II\text{-}III}$ sequence. It will be evident to those in the art, when in possession of the present disclosure, that modifications (*e.g.*, additions, deletions and substitutions) may be made to a particular peptide without substantially affecting the peptide's ability to act as a binding partner for SNAP-25, syntaxin, or a binding portion of either.

Additional preferred peptides include the human amino acid sequences known in Figure 11B that correspond to the sequences described above from Figure 11A. For example, preferred peptides include those which correspond to the amino acid sequences in Figure 11B from amino acid 717 to 1143, from 717 to 1036, from 717 to 962, from 717 to 858, and from 772 to 858. The above discussion regarding variations on the preferred peptides from Figure 11A is similarly applicable to the preferred peptides from Figure 11B and is incorporated here by reference thereto. Other peptides with one or more additions, deletions or substitutions to the sequences described herein may be tested for syntaxin binding and compared to the results disclosed herein for certain preferred peptides. Based upon the results of tests of any other peptides, it will be readily apparent whether a particular peptide is suitable.

Similar to that described above for the calcium channel, an entire syntaxin or SNAP-25 protein may be used, but it is not required as portions of the sequence will suffice. For example, the present invention discloses peptides that are less than an entire syntaxin amino acid sequence, yet still interact specifically with N-type or P/Q-type calcium channels or portions thereof. An

example of such a peptide is the sequence from amino acids 181 to 288 in Figure 12. Peptides derived from or based upon native syntaxin or native SNAP-25 and variations (non-naturally occurring) of either are herein collectively termed "syntaxin-like peptides" and "SNAP-25-like peptides," respectively. Such peptides have the ability to interact specifically with N-type or P/Q-type calcium channels or binding portions of either. It will be evident to those in the art, when in possession of the present disclosure, that such peptides may be identified using appropriate assays, such as those described herein.

Presynaptic calcium channel peptides (native) and variations (non-naturally occurring) that possess the ability to bind syntaxin, SNAP-25, or a binding portion of either, are herein collectively termed "calcium channel-like peptides." It will be evident to those in the art, when in possession of the present disclosure, that such peptides may be identified using appropriate assays, such as those described herein. Calcium channel-like peptides may be used to screen for one or more compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles, as mediated by syntaxin or SNAP-25. Assays for screening for such compounds may take a variety of formats, including direct and indirect (*e.g.*, competition or inhibition). In one embodiment, a candidate compound is tested for the ability to bind to a calcium channel-like peptide. For example, a candidate compound may be contacted with such a peptide that contains a reporter group. The reaction conditions (*e.g.*, from about 1 min to 24 hr, at about 4°C to about 37°C, and a pH of about 6 to 8.5) are sufficient to permit binding between the candidate compound and the peptide if binding is going to occur. The presence of binding is based upon the detection of the reporter group in association with the candidate compound.

In another embodiment, a candidate compound is tested for the ability to inhibit the binding of a calcium channel-like peptide to a syntaxin-like peptide or a SNAP-25-like peptide (*i.e.*, a peptide selected from syntaxin peptides and variations therefrom, or SNAP-25 peptides and variations therefrom, that bind presynaptic calcium channel or calcium channel-like peptide). Any of these peptides may contain a reporter group to detect the binding between the peptides. Alternatively, for example, each peptide may contain a reporter group component that interact upon binding of the peptides. A candidate compound may be incubated simultaneously with both peptides. Alternatively, for example, a candidate compound is incubated with a calcium channel-like peptide to permit binding, if any, between the compound and the peptide. A syntaxin-like peptide or a SNAP-25-like peptide is then contacted

with the reaction mixture to permit binding between the calcium channel-like peptide and the syntaxin-like or SNAP-25-like peptide. It may be desirable to include calcium ion in the reaction mixture, *e.g.*, to optimize binding between the peptides. For example, calcium may be included at a concentration of about 10-25 μM. Where a candidate compound does not bind to the calcium channel-like peptide, the peptide will bind to the syntaxin-like or SNAP-25-like peptide to the same extent as where the compound is absent (*e.g.*, compound replaced in the first step with buffered solution). Thus, the presence of binding between the calcium channel-like peptide and the syntaxin-like or SNAP-25-like peptide is indicative that the candidate compound did not bind to the former peptide. However, where a candidate compound does bind to the calcium channel-like peptide, the peptide is no longer available to bind to the syntaxin-like or SNAP-25-like peptide. Thus, the absence of binding between the calcium channel-like peptide and the syntaxin-like or SNAP-25-like peptide is indicative that the candidate compound does bind to the former peptide. All of the above discussion is equally applicable where a syntaxin-like peptide is replaced with syntaxin (*i.e.*, a full length syntaxin protein) or a SNAP-25-like peptide is replaced with SNAP-25 (*i.e.*, a full length SNAP-25 protein).

Detection of binding between a compound and peptide or between two peptides may be accomplished by a variety of known techniques, including radioassays and enzyme linked assays. For detection purposes, a peptide can be directly labeled with a reporter group. Alternatively, a molecule (*e.g.*, an antibody) that binds to a peptide or candidate compound can possess a reporter group. The reporter group can include radioisotopes, fluorophores, enzymes, luminescers, or dye particles. These and other labels are well known in the art and are described, for example, in the following U.S. patents: 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402.

The methods described herein may be used in a fully or partially automated format for high through-put screening of candidate compounds. For example, peptides may be utilized in a 96-well plate assay format with a reporter group amenable to automated analysis of the results. For example, the reporter group can be chosen from a variety of enzymes, including horseradish peroxidase, beta-galactosidase, alkaline phosphatase and glucose oxidase. The results of the reaction between an enzyme and its added substrate can be read optically in a 96-well plate reader.

Alternative formats, labeling and in general other modifications of the assays described above are within the scope of those in the art.

The methods and compositions of the present invention have a variety of uses. A particularly preferred use of the present invention is to screen for compounds that differentially modulate transmitter release versus current flow via calcium channels. Compounds may be identified that inhibit neurotransmitter release without significantly affecting calcium influx. Although calcium would still enter through presynaptic calcium channels, transmitter release would not occur because the synaptic vesicles would not be properly docked to respond to the locally increased calcium concentration. Such compounds would effectively block release of neurotransmitters in the central or peripheral nervous system and be useful in neuroprotection from excitotoxicity in many clinical settings, including the treatment of stroke, cognitive deficit related to cardiac surgery, and neuronal damage during acute epileptic episodes.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Construction and Expression of Recombinant GST- and His-Fusion Proteins

GST-Syntaxin 1A fusion proteins were generated by cloning amplified portions of the gene corresponding to the full-length (2-289), N-terminal region (2-190) and C-terminal region (181-289) of rat syntaxin 1A (Yoshida et al., *J. Biol. Chem. 267*:24925-24928, 1992; Bennett et al., *Science 257*:255-259, 1992) were amplified by PCR. *Eco*RI and *Xho*I sites were included at the ends of the N-terminal and C-terminal oligonucleotide primers, respectively; stop codons were included in both oligonucleotides. The amplified material was then cloned into *Eco*RI/*Xho*I digested pGEX-4T expression vector (Pharmacia LKB Biotechnology) to obtain in-frame recombinant proteins fused to glutathione S-transferase (GST).

A GST fusion protein including full-length SNAP-25 was similarly prepared by ligating a EcoRI-XhoI fragment of SNAP-25 (Oyler et al., *J. Cell Biol. 109*:3039-3052, 1989; amplified by PCR with synthetic oligonucleotides including the appropriate restriction sites) into the pGEX4T vector (Pharmacia LKB).

His-fusion proteins of α_{1B} were generated by amplification from oligonucleotides flanking a series of cytoplasmic domains of the $\alpha 1$ subunit of rat class B N-type calcium channels and containing appropriate restriction sites and in-frame stop codons. Polymerase chain reaction was performed using $\alpha 1_B$ cDNA (rbB-1) as a template (Dubel et al., *Proc. Natl. Acad. Sci. USA 89*:5058-5062, 1992) to amplify the appropriate DNA fragments. The amplified products were directionally cloned into the pTrcHis C expression vector (Invitrogen) that codes for a stretch of 6 histidine residues immediately following the initiator codon. The His-fusion proteins containing various cytoplasmic domains/loops of calcium channel $\alpha 1_B$ were as follows: the cytoplasmic amino-terminal, His-NT (41-94); the loops between domains I and II, His-L_{I-II} (357-483); the loops between domains II and III, His-L_{II-III} (718-1145); the loops between domains III and IV, His-L_{III-IV} (1418-1474); the cytoplasmic carboxyl-terminal I, His-CT-1 (1712-2068) and II, His-CT-2 (2044-2336); fragments between domains II and III, His-L_{II-III} (718-859), His-L_{II-III} (832-963), His-L_{II-III} (940-1051), His-L_{II-III} (1027-1145), His-L_{II-III} (718-825), His-L_{II-III} (744-859), His-L_{II-III} (773-859); the cytoplasmic loops between domains II and III of $\alpha 1_A$ (723-868) and $\alpha 1_S$ (670-800). All constructs were verified by determining the DNA sequence.

Constructs were transformed into a protease-deficient strain, BL26 (Novagen). Fusion protein expression was obtained following the basic protocol of Smith and Johnson (*Gene 67*:31-40, 1988). In brief, fresh overnight cultures were diluted 1:10 in YT medium containing 100 μg/ml ampicillin and 2% glucose and incubated for 4 hr at 37°C with shaking. After 2 hr of growth, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Bacterial cells were pelleted by centrifugation at 5000 x g for 10 min at 4°C and resuspended in PBS buffer. The bacteria were lysed by mild sonication and solubilized by the addition of Triton X-100 to final concentration of 1% followed by incubation for 30 min on ice.

A hBI clone was isolated by screening a cDNA library generated from the human small cell lung carcinoma line, SCC-9 (Oguro-Okano et al., *Mayo Clinic Proceedings 67*:1150-1159, 1992). Briefly, ~500,000 cDNAs were screened with a randomly primed ^{32}P-radiolabelled 1.9 kb Eco RI fragment from the rat brain class A cDNA, rbA-73 (Snutch et al., *Proc. Natl. Acad. USA 87*:3391-3395, 1990). Hybridization was carried out overnight at 62°C in 5X SSPE (1X SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.4), 0.3% SDS, 0.2 mg/ml denatured salmon sperm DNA. Filters were washed 4 times for 20 min in 0.5X SSPE at 60°C. A human brain BI clone was isolated

by screening a human hippocampus cDNA library (Stratagene, La Jolla, CA) with a ^{32}P-radiolabelled 1.3 kb Eco RV - Sst I fragment of the small cell lung carcinoma cDNA. DNA sequencing was performed on double-strand plasmid DNA with modified T7 polymerase (Sequenase 2.0, USB Corp., Cleveland, OH).

His-fusion proteins of α_{1A} were generated using cDNAs encoding the rat brain rbA-126 isoform (Starr et al., *Proc. Natl. Acad. Sci. USA 88*:5621-5625, 1991) and a human homolog of the BI-1 isoform (see above) from a small cell lung carcinoma cell line (Oguro-Okano et al. *Mayo Clinic Proceedings 67*:1150-1159, 1992) as a template and synthetic oligonucleotides with overhanging restriction sites as primers in a polymerase chain reaction. The amplified products were directionally cloned into the pTrcHis expression vector (Invitrogen) to yield cDNAs expressing the His-fusion proteins rbA 724-869, rbA 844-981, rbA 724-981, hBI 722-895, hBI 843-1036, and hBI 722-1036. Constructions of full-length GST-syntaxin 1A, GST-syntaxin 1A N-terminus, GST-syntaxin 1A C-terminus and His-fusion proteins of the N-type Ca^{++} channel were performed as described above. All constructs were verified by DNA sequence analysis. The protease-deficient Escherichia coli strain BL26 (Novagen) was used for expression following standard protocols (Smith and Johnson, *Gene 67*:31-40, 1988). Briefly, fresh overnight cultures were diluted 1:10 in LB medium containing ampicillin (100 μg/ml), incubated for 90 min at 37°C with shaking and induced upon addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 2-4 h growth, bacterial cultures were pelleted by centrifugation at 5000 x g for 10 min at 4°C and resuspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3) containing protease inhibitors (pepstatin, aprotinin, leupeptin (each at 4 μg/ml), 0.4 μM phenylmethylsulfonyl fluoride). The bacteria were then lysed by mild sonication, solubilized by adding Triton X-100 to a final concentration of 1 % and incubated for 30 min on ice. The suspension was centrifuged at 15,000 rpm for 15 min and the supernatant stored in aliquots at -20°C. The amount of each fusion protein in the supernatant was estimated with a standard curve relating the intensity of the immunoblotting signal to the amount of a standard fusion protein (T7 gene *10*, Novagen) applied. The pixel values for the Ca^{++} channel fusion proteins used were as follows: rbB 718-859, 1288; rbB 832-963, 1077; rbB 718-963, 405; rbA 724-869, 856; rbA 844-981, 274; rbA 724-981, 1117; BI 722-895, 1258; BI 843-1036, 321; BI 722-1036, 1154.

Example 2
Screening of His-Fusion Proteins for Binding to GST-Syntaxin

Binding of the cytoplasmic domains/loops of calcium channels to GST-syntaxin fusion proteins was assayed. Approximately 2 μg of GST-syntaxin fusion proteins or GST alone was bound to glutathione-Sepharose 4B beads (Pharmacia) in PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing 0.5% Triton X-100, 4 μg/ml pepstatin, 4 μg/ml aprotinin, 4 μg/ml leupeptin and 0.4 μM phenylmethylsulfonyl fluoride. The mixture was incubated at 4°C for 1 hour with constant agitation. Following incubation, the beads were washed with PBS to remove uncoupled GST fusion proteins. Glutathione-Sepharose beads preincubated with similar amounts of GST-syntaxin or GST were added to the lysates containing 5-10 μg His-fusion proteins of the various cytoplasmic domains/loops of calcium channels and incubated with gentle mixing for 3 hr at 4°C. Following incubation, beads were washed three times in ice-cold PBS with 0.1% Triton X-100, three times in 50 mM Tris-HCl, pH 8.0/140 mM NaCl/0, 1% Triton X-100, and once in 50 mM Tris-HCl, pH 8.0.

Bound fusion proteins were eluted with 50 mM Tris-HCl, pH 8.0/15 mM glutathione for 20 min with gentle mixing and eluates were separated from the beads by centrifugation at 10,000 x g for 1 minute. Fusion proteins were electrophoresed on SDS/PAGE and transferred to nitrocellulose. Specific fusion proteins were detected by an ECL kit (Amersham) using either an anti-GST or T7-Tag antibody (Novagen). The T7-Tag antibody is a mouse monoclonal directed against the 12 amino acid leader peptide in the N-terminal of His-fusion proteins. To assess the quantity and quality of the His-fusion proteins used in binding assays, approximately 10% amount of lysates was examined by SDS/PAGE and immunoblotting. The amount of each fusion protein in the binding assays was estimated with a standard curve relating the intensity of the immunoblotting signal to the amount of a standard fusion protein.

Interaction of His-L_{II-III} (718-1145) with GST-syntaxin fusion protein was observed as a specific band as illustrated in Figure 2 (middle lane, GST-syn). In contrast, no interaction was detected with GST alone (Figure 2, right lane). All other His-fusion proteins containing the cytoplasmic loops, as well as the N- and C-terminal of $\alpha 1_B$ failed to demonstrate specific interaction with GST-syntaxin (Figure 2), even though comparable quantities of the

expressed proteins were analyzed. These results suggest that there is a specific interaction between the cytoplasmic loop connecting domains II and III of $\alpha 1_B$ and syntaxin 1A.

In order to investigate the minimum sequence requirements for binding to syntaxin 1A, a series of His-fusion proteins covering various lengths of L_{II-III} were generated and analyzed for binding (Figure 3). As shown in Figure 4, the amino-terminal 142 amino acids (718-859) from L_{II-III} are sufficient for binding to syntaxin 1A. Larger quantities of fusion proteins containing the remaining 286 amino acids (residues 860-1145) located in L_{II-III} do not interact detectably with syntaxin 1A.

Further analysis of fusion proteins containing overlapping portions of the segment from residue 718 to 859 (Figure 3) indicated that a sequence of 87 amino acids (773-859) of $\alpha 1_B$ was sufficient for interaction with syntaxin 1A (Figure 4, right column). In contrast, the fusion peptides L_{II-III} (718-785) and L_{II-III} (744-825) do not interact specifically with syntaxin since no interaction was observed when they were incubated with the GST-syntaxin matrix (Figure 4, middle columns). Thus, the first 56 residues (718-773) of L_{II-III} are unlikely to be required for the syntaxin-binding site since His-L_{II-III} (718-785) did not show any binding activity while His-L_{II-III} (773-859) retained the full binding of the longer fusion peptide His-L_{II-III} (718-859).

To test whether $\alpha 1_A$ shares a similar syntaxin binding activity to $\alpha 1_B$, His-fusion proteins containing the corresponding region (residues 723-868) of the cytoplasmic loop L_{II-III} from $\alpha 1_A$ (Starr et al., *Proc. Natl. Acad. Sci. USA* *88*:5621-5625, 1991) were constructed. As a control, a His-fusion protein covering the entire L_{II-III} loop (residues 670-800) of the rabbit skeletal muscle L-type calcium channel ($\alpha 1_S$) (Tanabe et al., *Nature* *328*:313-318, 1987) was included in parallel syntaxin-binding assays. As shown in Figure 8, while the sequence (718-859) from $\alpha 1_B$ binds to GST-syntaxin, there is no detectable interaction between the corresponding region from $\alpha 1_A$ or $\alpha 1_S$ in the syntaxin-binding region. These results indicate that these weakly conserved regions of the cytoplasmic loop L_{II-III} from class A and class S calcium channels do not bind to syntaxin.

Example 3

Partial Purification of Rat Class B N-Type Calcium Channels

Brain calcium channels were partially purified as previously described (Westenbroek et al., *Neuron* 9:1099-1115, 1992). Briefly, fifteen rat brains cortices from three-week-old Sprague-Dawley rats were homogenized in 130 ml of 320 mM sucrose, 5 mM Tris pH 7.4 and protease inhibitors (1 μg/ml each pepstain A, leupeptin, and aprotinin, 0.2 mM phenyl methanesulfonyl fluoride, and 0.1 mg/ml benzamidine) by 10 strikes with a glass-Teflon homogenizer. After a short centrifugation (5000 rpm, 2 minutes, SS34-rotor), the membranes contained in the supernatant were pelleted (42,000 rpm, 1 hour, in Ti45 rotor) and solubilized in 230 ml of 1.2% digitonin in PBS (150 mM NaCl, 300 mM KCl, and 10 mM sodium phosphate buffer (pH 7.4) for 15 min on ice. Unsolubilized material was sedimented by centrifugation as before, and the supernatant was slowly poured over a 20 ml wheat germ agglutinin (WGA) Sepharose column (50 ml/hr.). The column was washed with 300 ml of 0.1% digitonin, 75 mM NaCl, 50 mM sodium phosphate, 10 mM Tris-HCl (pH 7.4) at a flow rate of 50 ml/hr.

Bound calcium channels were eluted with 100 mM N-acetyl-D-glucosamine in the same buffer at a flow rate of 50 ml/hr. Two ml fractions were collected, frozen and stored at -80°C. About 50% of the solubilized N-type calcium channels were specifically bound and eluted from WGA-Sepharose under these experimental conditions (Westenbroek et al., *Neuron* 9:1099-1115, 1992).

Example 4

Immunoprecipitation

The WGA extraction fraction was labeled for 2 hr on ice with 500 fmol [^{125}I]Tyr^{22}-ω-CTx-GVIA (NEN-Dupont), diluted 10-fold with PBS, and incubated for 2 hr at 4°C with either anti-CNB-1 antibody, which is directed against residues 851-867 of the α1 subunit of rat brain class B N-type calcium channel (Westenbroek et al., *Neuron* 9:1099-1115, 1992) or mAb 10H5, an anti-syntaxin antibody (Yoshida et al., *J. Biol. Chem.* 267:24925-24928, 1992). Immune complexes were recovered by the addition of 4 mg of protein A-Sepharose 4B swollen in TBS, rotation for 1 hour, and centrifugation. After three washes in PBS, immunoprecipitated radioactivity was counted.

Anti-CNB1 antibodies immunoprecipitated 85% ± 6% (n = 3) of ω-CTx receptors were also immunoprecipitated by anti-syntaxin antibody mAb10H5, but not by control mouse IgG (1.6% ± 0.4%, n = 3), indicating that only a small fraction of N-type channels remains associated with syntaxin after treatment with digitonin and subsequent WGA-Sepharose column purification. Thus, this procedure provides N-type calcium channels containing $\alpha 1_B$ with only 12% of their syntaxin-binding sites occupied.

Example 5

Inhibition of Binding of [^{125}I]-ω-CTx-GVIA-Labeled N-Type Calcium Channels by the His-L_{II-III} (773-859) Peptide

Approximately 2 μg of either GST-syntaxin or GST were coupled to glutathione-Sepharose 4B beads in PBS/0.5% Triton X-100 for 1 hour at 4°C. After removal of unbound proteins by washing with PBS/0.1% Triton X-100 for three times, an equal amount of lysate containing either His-L_{II-III} (773-859) or His-L_{II-III} (1027-1145), as a non-inhibitor control, was added to the beads. After a 1 hr incubation, an equal amount of cpm of [^{125}I]-ω-CTx-GVIA-labeled N-type calcium channel was added to each reaction mixture. After a further 3 hour incubation, the beads were washed three times with PBS and the amount of bound receptor was assessed by direct counting.

As shown in Figure 10B, GST-syntaxin bound 3276 ± 191 cpm (n = 3) of labeled N-type channels, whereas GST alone bound only 549 ± 74 cpm (n = 3). This observation shows directly that syntaxin can bind N-type calcium channels *in vitro* and strengthens the conclusion that N-type calcium channels are tightly associated with syntaxin (Bennett et al., *Science 257*:255-259, 1992; Lévêque et al., *J. Biol. Chem. 269*:6306-6312, 1994; Yoshida et al., *J. Biol. Chem. 267*:24925-24928, 1992; O'Conner et al., *FEBS Lett. 326*:255-261, 1993). These results also confirm that GST-syntaxin fusion proteins attached to an affinity matrix maintain the binding activity for calcium channels.

In order to demonstrate that the binding sequence identified in *in vitro* binding assays represents the high affinity syntaxin-binding site in $\alpha 1_B$, the ability of the 87-amino-acid binding peptide to compete for binding of native N-type calcium channels to GST-syntaxin was analyzed. Peptide competition analysis demonstrated that the peptide, His-L_{II-III} (773-859), specifically competed for the binding of N-type calcium channels to GST-syntaxin (Figure 10C). In three independent experiments, a 78% ± 12% reduction in the

specific binding of ω-CTx-labeled N-type calcium channels to GST-syntaxin in the presence of lysate containing peptide His-L_{II-III} (773-859) was observed. Only a 10% ± 8% reduction was seen in the presence of a control lysate containing approximately the same amounts of the peptide His-L_{II-III} (1027-1145), which contains the carboxyl terminus of L_{II-III}.

Example 6

Binding Assays and Immunoblot Analysis

A. *In Vitro* Binding Assays

GST fusion proteins (150 pmol) were bound to glutathione agarose beads (30 μl, Pharmacia LKB) in PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3) containing 0.1 % Triton X-100, pepstatin, aprotinin, leupeptin (each at 4 μg/ml), 0.4 μM phenylmethylsulfonyl fluoride. The mixture was incubated at 4°C for 1 hr with constant agitation. The beads were then washed with PBS and incubated with identical concentrations of expressed His-fusion proteins of different Ca^{++} channels for 3 hr at 4°C. Beads were then washed three times in ice-cold PBS/0.1 % Triton X-100 and two times in 50 mM Tris-HCl pH 8/0.1 % Triton X-100. Bound proteins were eluted from the beads by competition with 15 mM reduced glutathione/50 mM Tris-HCl pH 8 (20 μl) for 20 min with gentle mixing. Eluates were separated from the beads by centrifugation at 10,000 x g for 1 min, mixed with 10 μl 3x Tricine sample buffer and boiled for 2 min.

B. Immunoblot Analysis

Proteins were separated on 10-20 % Tricine gradient gels (Novex) and transferred overnight to nitrocellulose (0.45 μm, Schleicher & Schuell). The membrane was then incubated with 5 % powdered milk in TBS (10 mM Tris pH 8, 150 mM NaCl, and 0.1 % Tween 20) for 1 h at room temperature. Blots were washed three times in TBS and incubated with Anti-T7.Tag monoclonal antibody (1:10,000, Novagen) for 1 h at RT. Following three washes with TBS, incubation with Anti-mouse IgG conjugated with horseradish peroxidase (1:10,000, Amersham) was performed. Blots were then washed for 1 h with TBS and the immunoreactive bands were visualized by enhanced chemoluminescence (ECL system, Amersham). Bound antisera were quantitated by phosphorimaging (Molecular Dynamics). The pixel values for Figure 14 were rbB 718-859, 45; rbB 832-963, 751; and rbB 718-963, 1646.

Example 7

Identification of a Second Syntaxin-binding Segment in the α_{1B} Subunit of N-type Ca^{++} Channels

As shown above, a syntaxin-binding site on the N-type Ca^{++} channel was identified using *in vitro* binding assays. Deletion analysis revealed a minimum requirement of 87 amino acids in the intracellular loop L_{II-III} (rbB 773-859, Figure 13) to maintain binding activity. However, the affinity of Ca^{++} channel fusion proteins for syntaxin is progressively reduced as their size is decreased toward the minimum sequence of 87 amino acid residues. Therefore, binding studies with larger fusion proteins of L_{II-III} were performed to identify additional regions which influence the affinity of syntaxin binding. In order to be able to draw conclusions about the binding affinities, equal concentrations of the fusion proteins were used in each binding assay by relating the intensity of the immunoblotting signal of each fusion protein lysate to a standard curve established for a standard fusion protein. As shown in Figure 14, rbB 718-963 binds with approximately 40-fold higher affinity to syntaxin than rbB 718-859. The contribution of rbB 832-963 can account for this difference in affinity as the sum of signal intensities of rbB 718-859 and rbB 832-963 is approximately equal to the intensity of rbB 718-963. No binding to GST alone was detected for all three fusion proteins (Figure 14, right lanes), demonstrating the specificity of the interactions. Therefore, the syntaxin-binding site of N-type Ca^{++} channel is composed of two different regions of the intracellular loop L_{II-III}.

Example 8

Isoforms of α_{1A}

Screening of cDNA libraries generated from human hippocampus and a small cell carcinoma cell line (Oguro-Okano et al., *Mayo Clinic Proceedings 67*:1150-1159, 1992) resulted in the isolation of clones encoding a human homologue of the BI isoform of α_{1A}. The predicted amino acid sequences of the human hippocampal and small cell carcinoma BI isoforms were identical in the L_{II-III} region and share 92% and 82% identity with the rabbit BI and rat rbA isoforms, respectively (Figure 15). Of particular note, alignment of the three sequences shows that relative to rbA, the human α_{1A} isoform contains several insertions of nearly identical sequence at the same positions as those of

rabbit BI, indicating that the human α_{1A} cDNAs correspond to the BI isoform. These observations support the hypothesis that BI and rbA are different isoforms of α_{1A} which may be present in all three species.

Based on the binding behavior of the α_{1B} fusion proteins described above, corresponding histidine-tagged (His)-fusion proteins of the L_{II-III} regions of the human BI (hBI) isoform and the rat rbA isoforms were generated. Recombinant GST-syntaxin or GST alone was bound to glutathione-Sepharose beads and incubated with equal concentrations of the six α_{1A} His-fusion proteins. Immunoblot detection revealed that the largest fusion protein of the hBI isoform (hBI 722-1036) is able to bind with high affinity to GST-syntaxin. For the α_{1A} fusion proteins, no binding to GST alone was detected. Titration studies demonstrated that, under our binding conditions, half-maximal saturation occurs at approximately 2 μM concentration of hBI 722-1036. These results with the BI isoform of α_{1A} are in contrast to the binding studies with the N-type channel where the binding contribution of the two smaller fusion proteins rbB 718-859 and rbB 832-963 are additive (Figure 14), and the affinity of the corresponding fusion protein (rbB 718-963) is approximately 10-fold greater (0.2 μM). It appears that the syntaxin-binding site on the BI isoform of α_{1A} has lower affinity than the one on the N-type channel and, therefore, requires the entire binding region to attain an active conformation.

Example 9

Both α_{1A} and α_{1B} Bind to the C-Terminal One-Third of Syntaxin

Syntaxin possesses three heptad repeats, two in its amino terminus (aa 30-64 and aa 68-112) and one in its carboxy terminus (aa 189-231) (Inoue et al., *J. Biol. Chem. 267*:10613-10619, 1992). As shown above, the rbB 718-859 fusion protein of α_{1B} binds to a GST-syntaxin deletion construct which contained only the C-terminal one-third (aa 181-288) of syntaxin 1A. However, it remained possible that the second syntaxin-binding segment of α_{1B} (rbB 832-963, Figure 1B) binds to another site on syntaxin, possibly the N-terminal heptad repeats. Binding studies with rbB 718-859, rbB 832-963 and rbB 718-963 fusion proteins of α_{1B} and the C-terminal segment of syntaxin were performed. As shown in Figure 16, all three fusion proteins bind to the C-terminal one-third of syntaxin 1A (aa 181-288). Conversely, no binding to GST-syntaxin-NT (aa 2-190) or GST alone could be detected, suggesting that both of the syntaxin-

binding regions of N-type Ca^{++} channels interact with a C-terminal site on syntaxin near the intracellular surface of the presynaptic plasma membrane.

Similar to full-length syntaxin 1A, only the largest fusion protein of the BI isoform showed any detectable binding to syntaxin fragments. It binds specifically to the C-terminal one-third of syntaxin 1A (aa 181-288) whereas no binding to GST-syntaxin-NT or GST alone occurred.

Example 10

α_{1A} and α_{1B} Compete for the Same Binding Region on Syntaxin 1A

Because both α_{1B} and the BI isoform of α_{1A} bound to the C-terminal one-third of syntaxin 1A, it was investigated whether both occupy the same binding site on syntaxin. Competition assays were performed with fusion proteins of α_{1B} and the BI isoform of α_{1A} present simultaneously in the binding solution. GST-syntaxin 1A was bound to glutathione-Sepharose beads and incubated with a constant concentration (5 μM) of hBI 722-1036 of α_{1A} and increasing concentrations of rbB 718-963 of α_{1B}. The signal intensity of hBI 722-1036 of the P/Q-type channel diminishes progressively while the signal intensity of rbB 718-963 of the N-type channel increases. These results demonstrate that P/Q-type and N-type channels indeed compete for the same binding region on syntaxin 1A.

Example 11

α_{1A} and α_{1B} Bind to the Presynaptic Protein SNAP-25

In order to elucidate whether the Ca^{++} channels interact with SNAP-25 (another presynaptic plasma membrane protein). a GST fusion protein of SNAP-25 was constructed as described in Example 1. GST-SNAP-25 was bound to glutathione-Sepharose beads and incubated with equal concentrations of the three α_{1B} fusion proteins and the six rbA and hBI α_{1A} fusion proteins. No binding to GST alone was detected, demonstrating the specificity of the interactions. In contrast, GST-SNAP-25 interacts with L_{II-III} of all three Ca^{++} channel types (Figure 17).

All three fusion proteins of the N-type channel (rbB 718-859, rbB 832-963, and rbB 718-963) bound to GST-SNAP-25. The signal intensities of rbB 718-859 and rbB 832-963 were comparable to the intensity of the rbB 718-

963 signal. These data demonstrate that the same two adjacent regions on the N-type channel which bind to syntaxin are also able to bind to SNAP-25.

The hBI 722-1036 fusion protein of the P/Q-type Ca^{++} channel shows the most intense immunoblot signal. Titration studies demonstrate half-maximal saturation at approximately 1-2 μM, comparable to that for syntaxin. The long exposure shown in Figure 17 reveals weak binding of hBI 843-1036, whereas no binding of hBI 722-895 is observed. These data support the hypothesis that both regions of the binding site on the BI isoform of α_{1A} are required for high affinity binding of presynaptic proteins.

In contrast to the BI isoform, the rbA 724-981 fusion protein shows weak binding to GST-SNAP-25. Nevertheless, this binding is specific because no binding to GST alone is observed. In addition, a fusion protein containing the entire intracellular loop of the rbA binds with comparable intensity. Although the binding is much weaker than the corresponding region of the BI isoform, these data suggest that the rbA isoform of the P/Q-type channel can also interact with proteins of the docking and fusion machinery. Evidently, the sequence differences in L_{II-III} between BI and rbA isoforms results in different binding affinities for SNAP-25.

Example 12

α_{1A} and α_{1B} Compete for the Same Binding Region on SNAP-25

In order to test if the binding regions on α_{1B} and the BI isoform of α_{1A} compete for binding to SNAP-25, a competition assay was performed with GST-SNAP-25 bound to glutathione-Sepharose beads. As shown in Figure 18, increasing concentrations of rbB 718-963 of α_{1B} displace hBI 722-1036 of α_{1A} from its binding site on SNAP-25. Evidently, α_{1A} and α_{1B} interact with identical or overlapping regions on SNAP-25 as well as on syntaxin.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually incorporated by reference.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

Claims

1. A method of screening for compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles, comprising the steps of:

(a) contacting a calcium channel-like peptide with a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound, wherein the peptide is able to bind syntaxin or SNAP-25; and

(b) detecting the presence or absence of binding between the peptide and the candidate compound, thereby determining whether the candidate compound bound to the peptide.

2. The method of claim 1 wherein step (a) further includes syntaxin or a syntaxin-like peptide under conditions sufficient to permit binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide; and step (b) comprises detecting the presence or absence of binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide. thereby determining whether the candidate compound inhibited the binding.

3. The method of claim 1 wherein step (b) comprises the steps of:

(b) adding syntaxin or a syntaxin-like peptide to the reaction mixture of the candidate compound and the calcium channel-like peptide of step (a) under conditions sufficient to permit binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide; and

(c) detecting the presence or absence of binding between the calcium channel-like peptide and the syntaxin or the syntaxin-like peptide, thereby determining whether the candidate compound inhibited the binding.

4. The method of claim 1 wherein step (a) further includes SNAP-25 or a SNAP-25-like peptide under conditions sufficient to permit binding between the calcium channel-like peptide and the SNAP-25 or the SNAP-25-like peptide; and step (b) comprises detecting the presence or absence of binding between the calcium channel-like peptide and the SNAP-25 or the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

5. The method of claim 1 wherein step (b) comprises the steps of:

(b) adding SNAP-25 or a SNAP-25-like peptide to the reaction mixture of the candidate compound and the calcium channel-like peptide of step (a) under conditions sufficient to permit binding between the calcium channel-like peptide and the SNAP-25 or the SNAP-25-like peptide; and

(c) detecting the presence or absence of binding between the calcium channel-like peptide and the SNAP-25 or the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

6. The method of any one of claims 1-5, wherein the calcium channel-like peptide has at least 87 amino acid residues selected from amino acid 717 to amino acid 1143 of an α_1 subunit of a N-type or Q-type calcium channel.

7. A peptide consisting essentially of an amino acid sequence of between 246 to 424 amino acid residues in length, wherein said amino acid sequence contains an amino acid sequence having at least 60% sequence similarity with the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to arginine, amino acid 963.

8. A peptide consisting essentially of the amino acid sequence of Figure 11A from an amino acid positioned between glutamic acid, amino acid 718, and alanine, amino acid 773, to an amino acid positioned between aspartic acid, amino acid 859, and cysteine, amino acid 1141.

9. A peptide consisting essentially of the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to cysteine, amino acid 1141.

10. A peptide consisting essentially of the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to arginine, amino acid 963.

11. A peptide consisting essentially of the amino acid sequence of Figure 11A from glutamic acid, amino acid 718, to aspartic acid, amino acid 859.

12. A peptide consisting essentially of the amino acid sequence of Figure 11A from alanine, amino acid 773, to aspartic acid, amino acid 859.

13. A peptide consisting essentially of an amino acid sequence of between 246 to 427 amino acid residues in length, wherein said amino acid sequence contains an amino acid sequence having at least 60% sequence similarity with the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to arginine, amino acid 962.

14. A peptide consisting essentially of the amino acid sequence of Figure 11B from an amino acid positioned between glutamic acid, amino acid 717, and alanine, amino acid 772, to an amino acid positioned between aspartic acid, amino acid 858, and cysteine, amino acid 1143.

15. A peptide consisting essentially of the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to cysteine, amino acid 1143.

16. A peptide consisting essentially of the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to threonine, amino acid 1036.

17. A peptide consisting essentially of the amino acid sequence of Figure 11B from glutamic acid, amino acid 717, to aspartic acid, amino acid 858.

18. A peptide consisting essentially of the amino acid sequence of Figure 11B from alanine, amino acid 772, to aspartic acid, amino acid 858.

19. A peptide consisting essentially of the amino acid sequence of hBI of Figure 15 from glutamic acid, amino acid 722, to arginine, amino acid 1036.

20. A peptide consisting essentially of the amino acid sequence of Figure 12 from isoleucine, amino acid 181, to glycine, amino acid 288.

21. A method of screening for compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles, comprising the steps of:

(a) contacting a peptide according to any one of claims 7-19 with a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound; and

(b) detecting the presence or absence of binding between the peptide and the candidate compound, thereby determining whether the candidate compound bound to the peptide.

22. A method of screening for compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles, comprising the steps of:

(a) incubating a candidate compound, a first peptide according to any one of claims 7-19, and syntaxin or a second peptide according to claim 20 under conditions sufficient to permit binding between the first peptide and syntaxin or between the first peptide and the second peptide; and

(b) detecting the presence or absence of binding between the first peptide and syntaxin or between the first peptide and the second peptide, thereby determining whether the candidate compound inhibited the binding.

23. A method of screening for compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles, comprising the steps of:

(a) incubating a first peptide according to any one of claims 7-19 and a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound, to form a reaction mixture;

(b) contacting syntaxin or a second peptide according to claim 20 with the reaction mixture under conditions sufficient to permit binding between the first peptide and syntaxin or between the first peptide and the second peptide; and

(c) detecting the presence or absence of binding between the first peptide and syntaxin or between the first peptide and the second peptide, thereby determining whether the candidate compound inhibited the binding.

24. A method of screening for compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles, comprising the steps of:

(a) incubating a candidate compound, a first peptide according to any one of claims 7-19, and SNAP-25 or a SNAP-25-like peptide under conditions sufficient to permit binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide; and

(b) detecting the presence or absence of binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

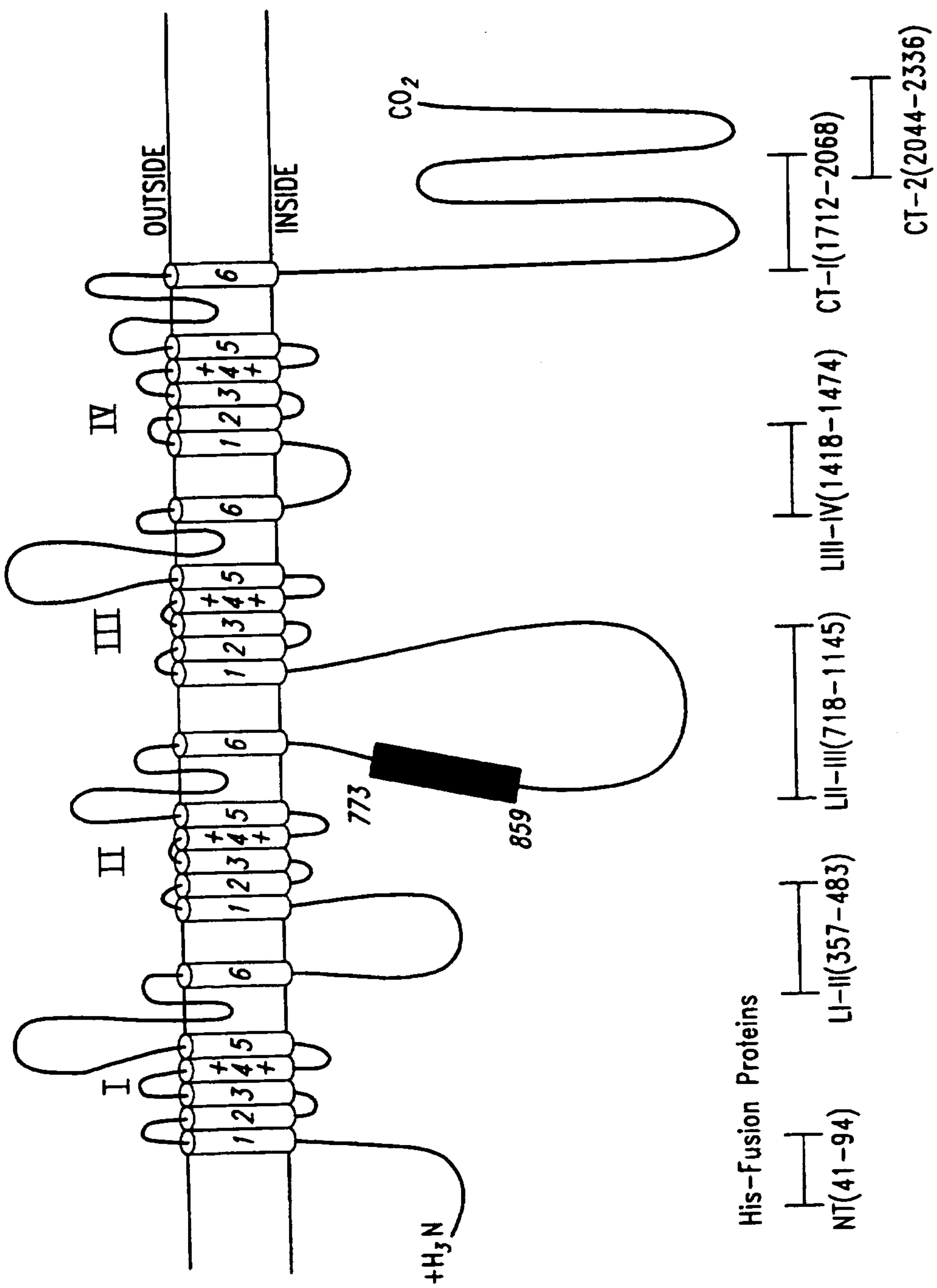
25. A method of screening for compounds that inhibit the interaction between presynaptic calcium channels and presynaptic vesicles, comprising the steps of:

(a) incubating a first peptide according to any one of claims 7-19 and a candidate compound under conditions sufficient to permit binding between the peptide and the candidate compound, to form a reaction mixture;

(b) contacting SNAP-25 or a SNAP-25-like peptide with the reaction mixture under conditions sufficient to permit binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide: and

(c) detecting the presence or absence of binding between the first peptide and SNAP-25 or between the first peptide and the SNAP-25-like peptide, thereby determining whether the candidate compound inhibited the binding.

Figure 1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II
III
IV
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859
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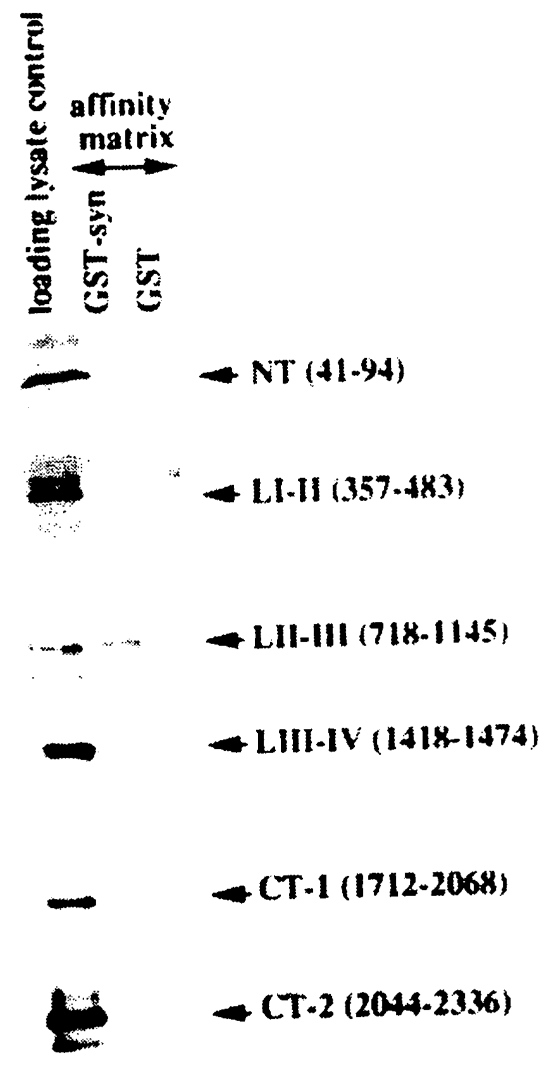
LI-II(357-483)
LII-III(718-1145)
LIII-IV(1418-1474)
CT-I(1712-2068)
CT-2(2044-2336)

Figure 2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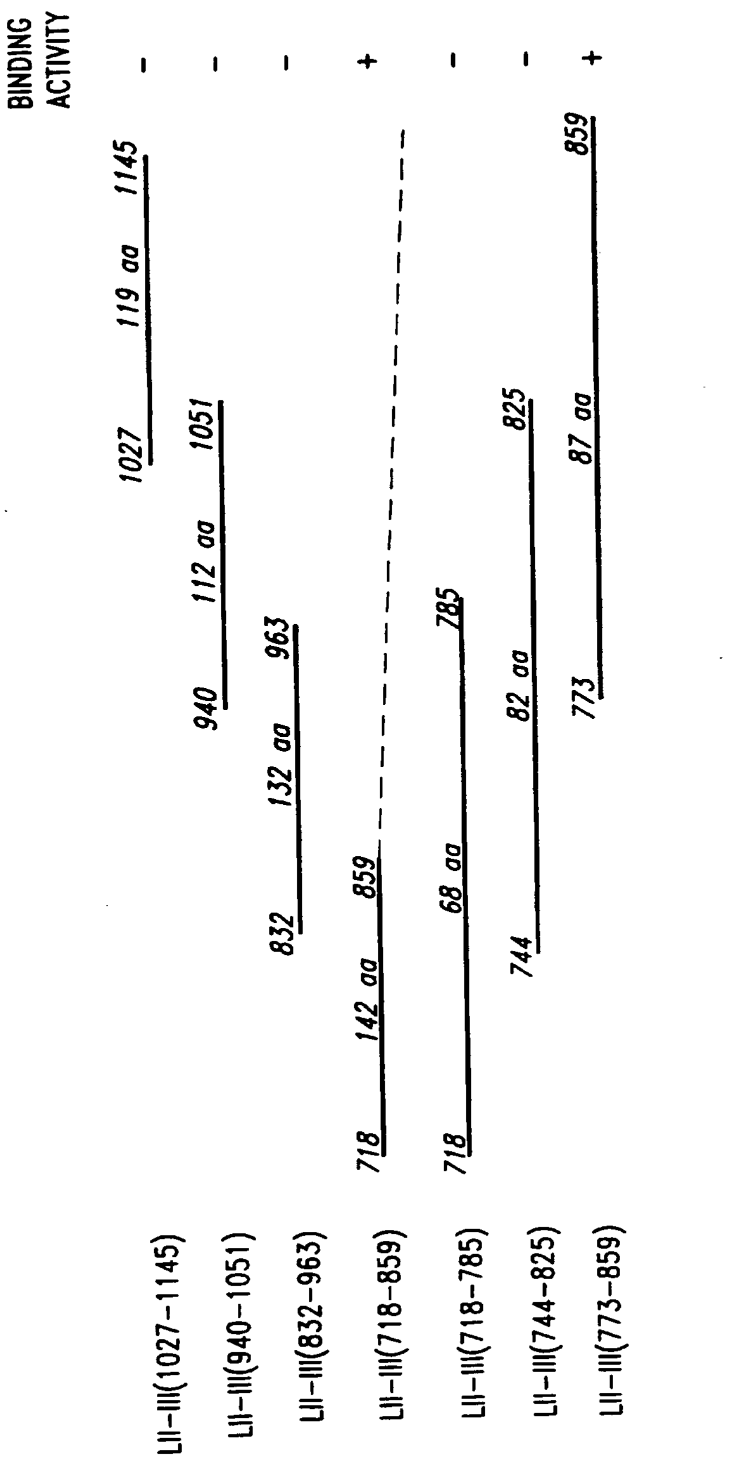
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1145
-
LII-III(940-1051)
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1051
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832
132 aa
963
-
LII-III(718-859)
718
142 aa
859
+
LII-III(718-785)
718
68 aa
785
-
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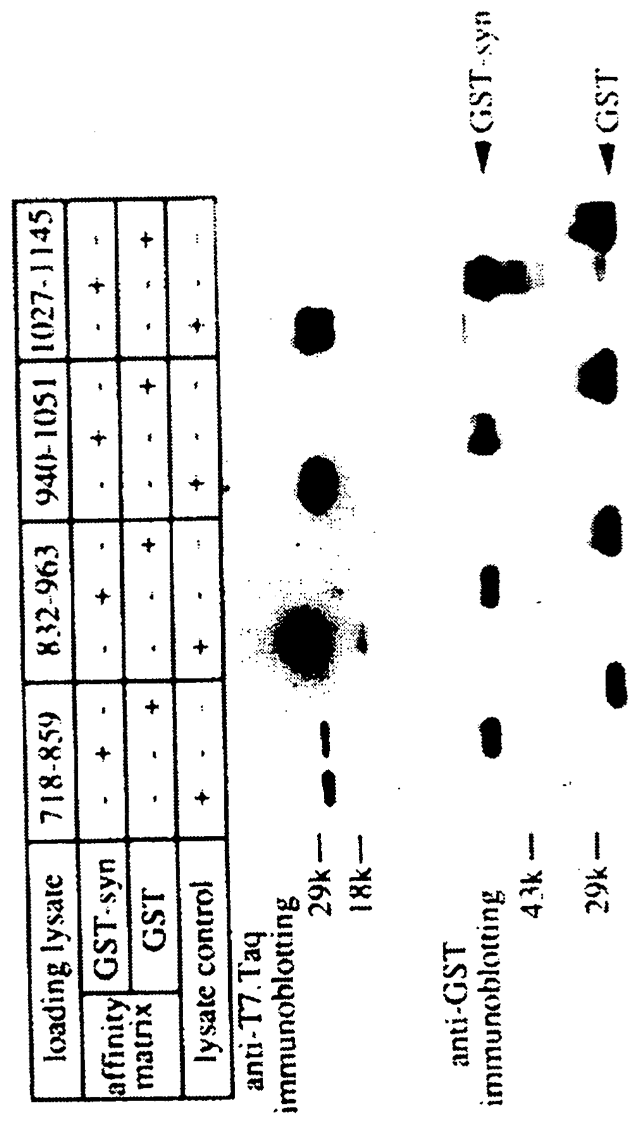
825
-
LII-III(773-859)
773
87 aa
859
+

Figure 3

Figure 4A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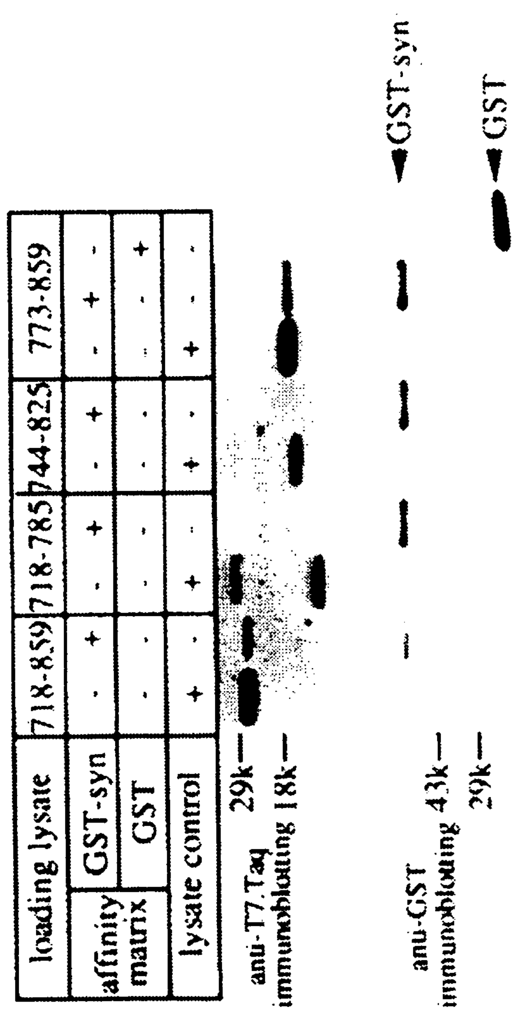
18k
anti-GST immunoblotting
43k
29k
GST-syn
GST

Figure 4B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773-859
affinity matrix
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lysate control
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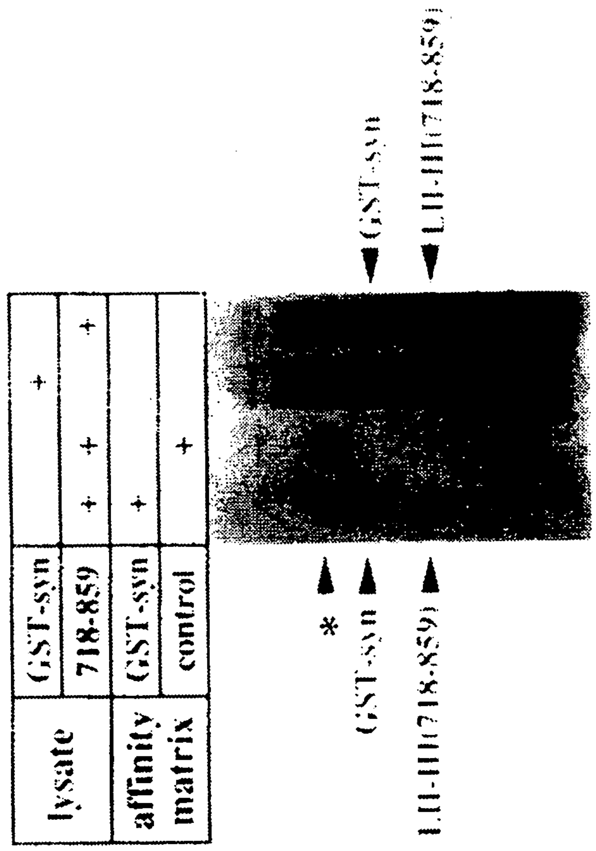
18k
anti-GST immunoblotting
43k
29k
GST-syn
GST

Figure 5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GST-syn
718-859
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control
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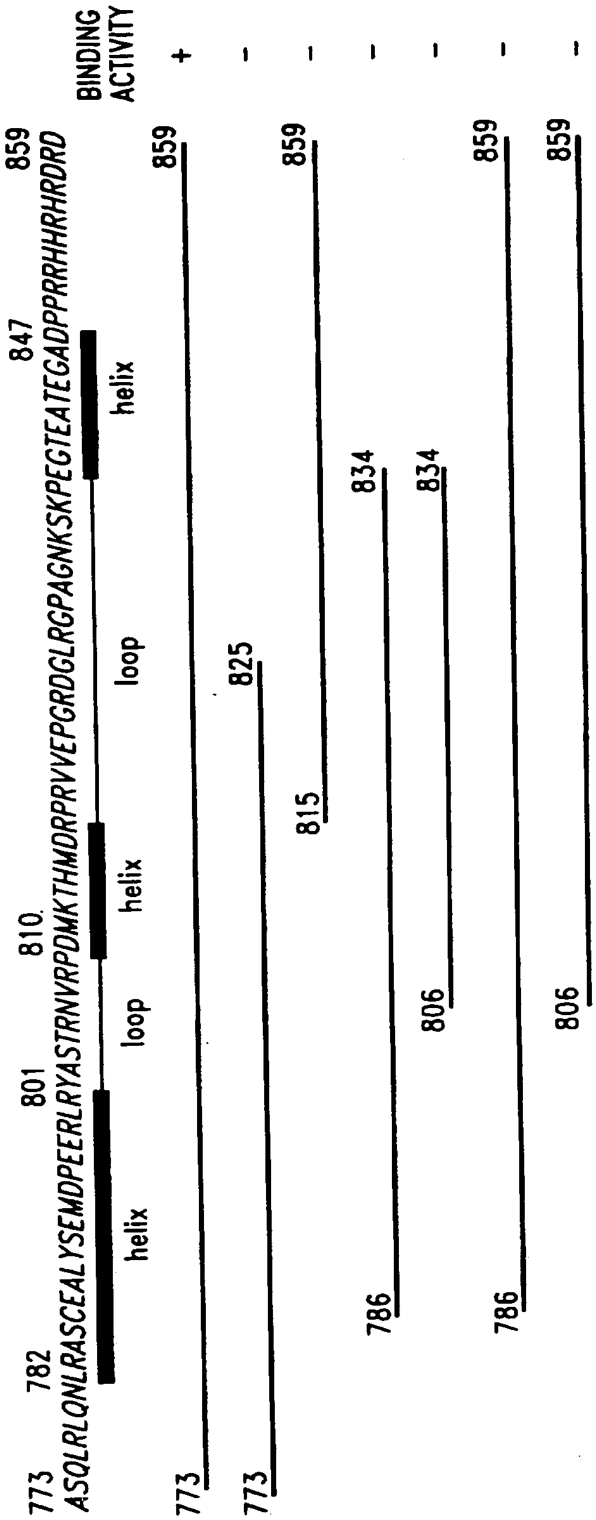
Figure 6



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loop
helix
loop
helix
BINDING ACTIVITY
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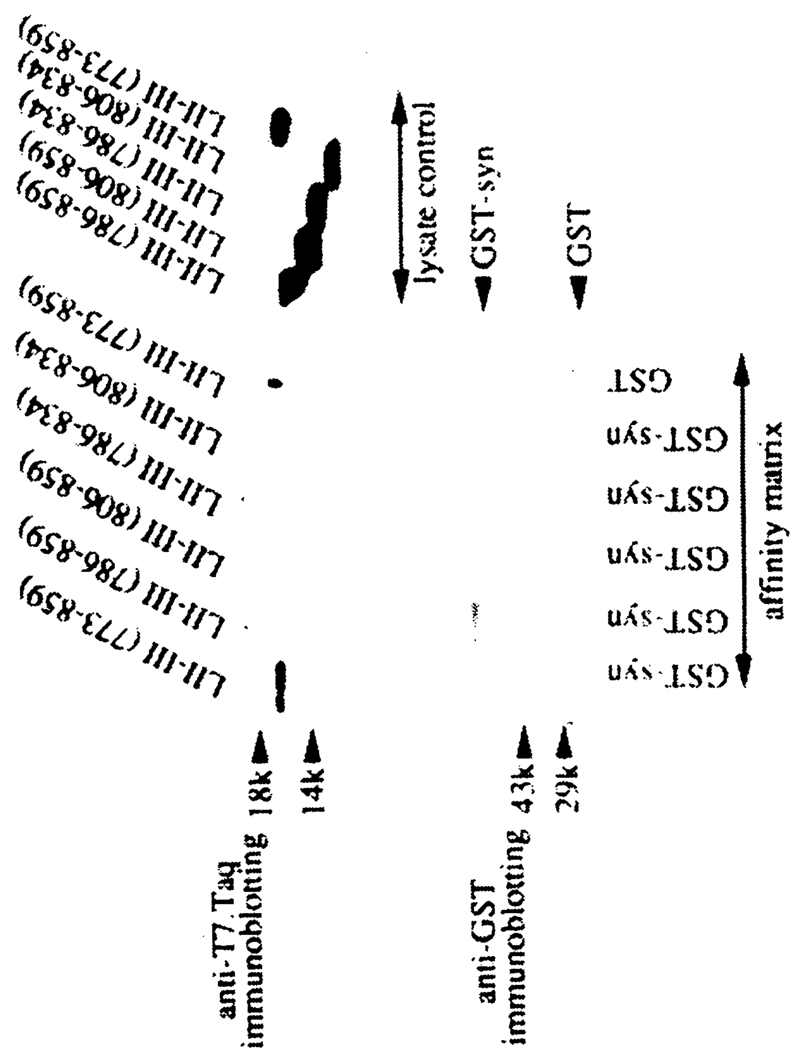
786 859 -
806 859 -

Figure 7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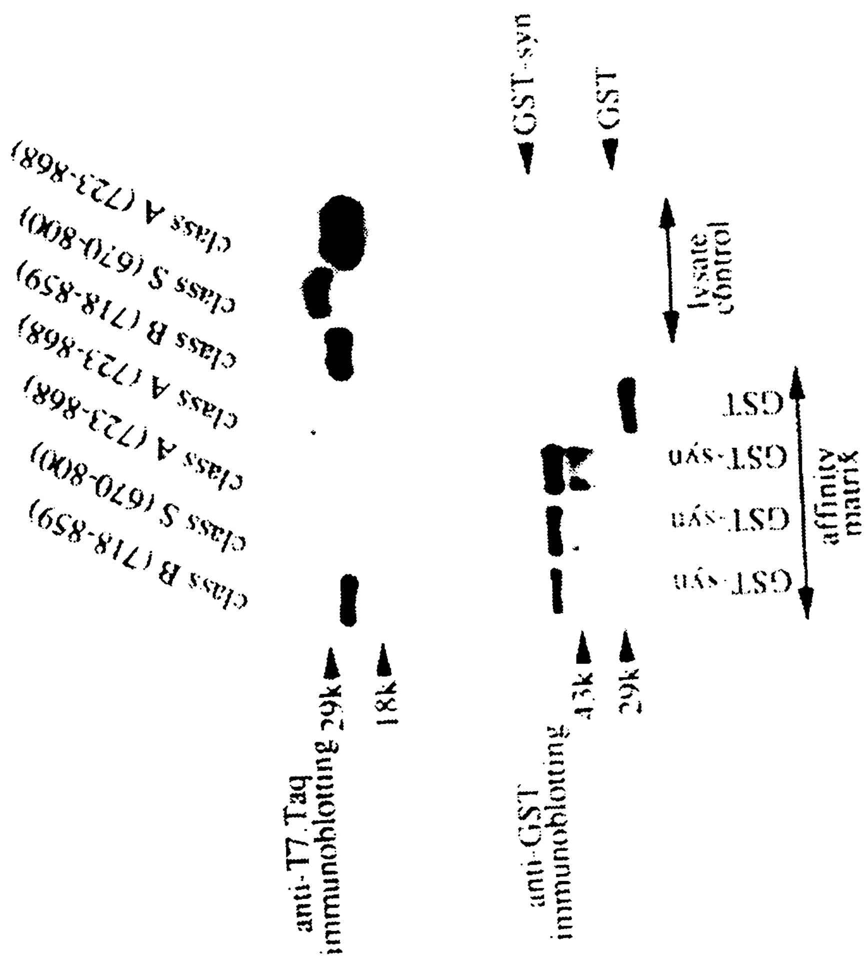
43k
29k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class S (670-800)
class B (718-859)
GST-syn
GST
lysate control
GST
GST-syn
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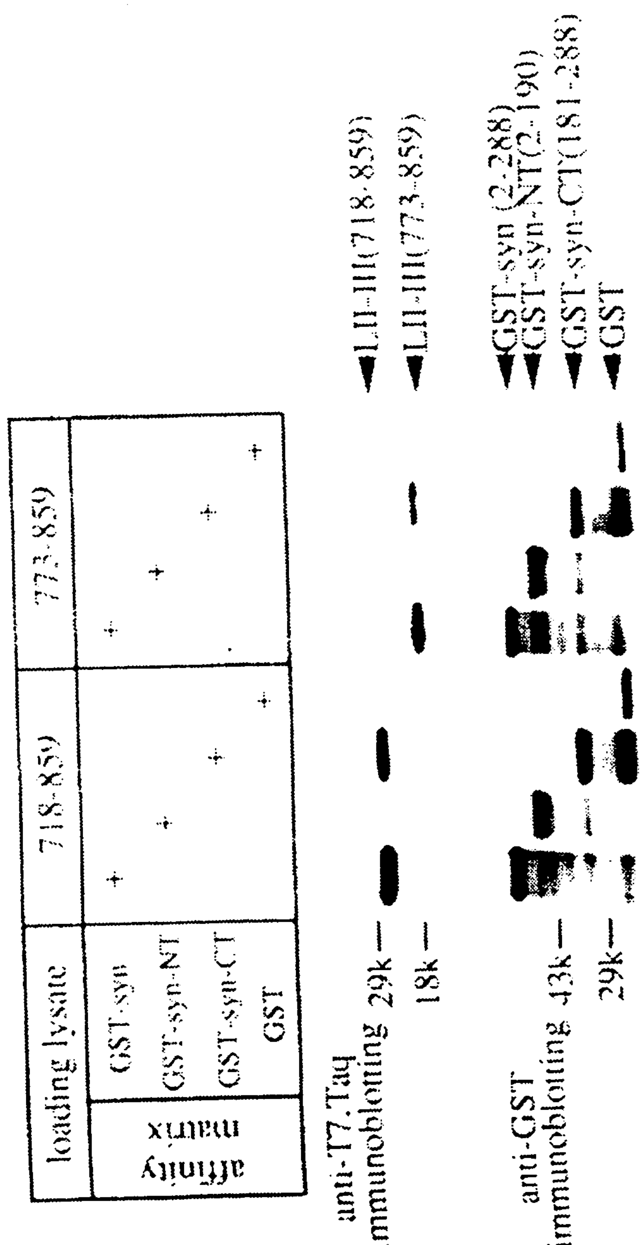
18k
anti-GST immunoblotting
43k
29k

Figure 8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773-859
affinity matrix
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GST
anti-T7.Taq immunoblotting
29k
18k
anti-GST immunoblotting
43k
29k
LII-III(718-859)
LII-III(773-859)
GST-syn (2-288)
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GST-syn-CT(181-288)
GST

Figure 9

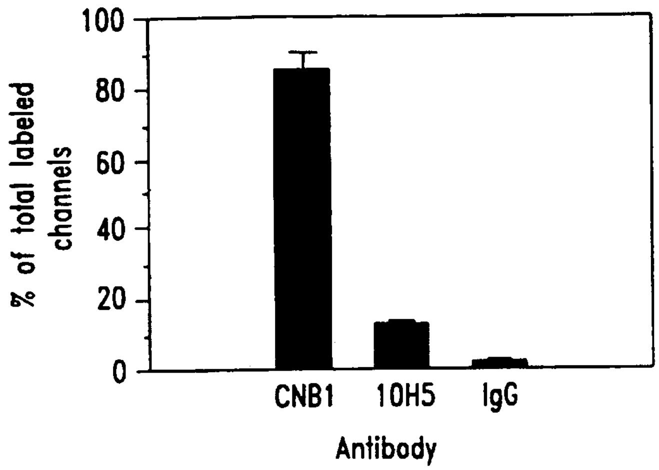

Figure 10A

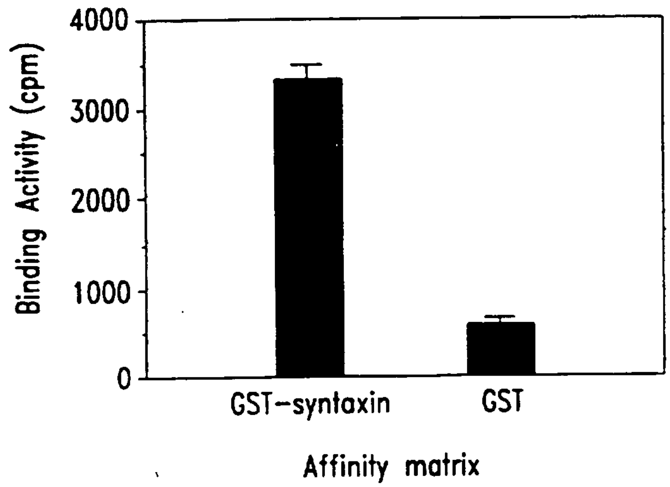

Figure 10B

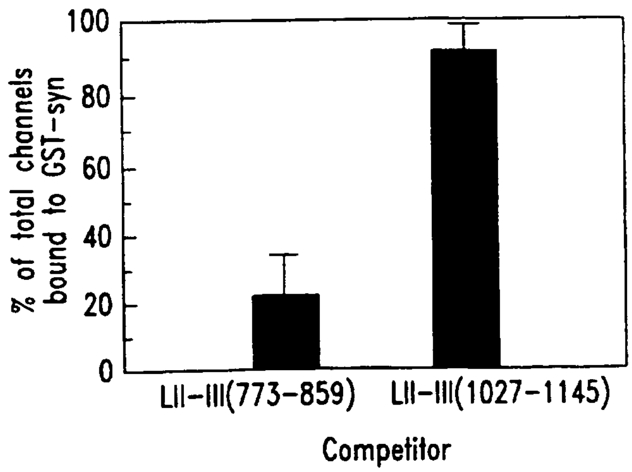

Figure 10C

Figure 11

```
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Fig. 11B -   710   .DNLANAQELTKDEEEMEEAANQKLALQKAKEVAEVSPMSAANISIAARQ   758

             760   QNSAKARSVWEQRASQLRLQNLRASCEALYSEMDPEERLRYASTRHVRPD   809
                   ||||||||||||||||||||||||||||||||||||||||:|.|||:|||
             759   QNSAKARSVWEQRASQLRLQNLRASCEALYSEMDPEERLRFATTRHLRPD   808

             810   MKTHMDRPLVVEPGRDGLRGPAGNKSKPEGTEATEGADPPRRHHRHRDRD   859
                   ||||:||||||| |||| |||.|.|.:||:.||.||.|||||||||||:|
             809   MKTHLDRPLVVELGRDGARGPVGGKARPEAAEAPEGVDPPRRHHRHRDKD   858

             860   KTSASTPAGGEQDRTDCPKAESTETGAREERARPRRSHSKEAPGADTQVR   909
                   |    |||:|:|||.:.|||||.|.||||||:||:|||||||:|: .:.|
             859   K....TPAAGDQDRAEAPKAESGEPGAREERPRPHRSHSKEAAGP.PEAR   903

             910   CER........SRRHHRRGSPEEATEREPRRHRAHRHAQDSSKEGKEGTA   951
                   :||        :||||||||||||.|||||||||||| ||.|||. ::
             904   SERGRGPGPEGGRRHHRRGSPEEAAEREPRRHRAHRH.QDPSKECAGA..   950

             952   PVLVPKGERRARHR.GPRTGPRETENSEEPTRRHRAKHKVPPTLEP....   996
                        ||||||||| |||.||||.|.:|||.|||||:||..|. |:
             951   .....KGERRARHRGGPRAGPREAESGEEPARRHRARHKAQPAHEAVEKE   995

             997   .PEREVAEKESNVVEGDKET..RNHQPKEPRCDLEAIAVTGVGSLHMLPS   1043
                    .|:|..|||.::||:|||.  |||||:||:||||. :...||.:| |||
             996   TTEKEATEKEAEIVEADKEKELRNHQPREPHCDLETSGTVTVGPMHTLPS   1045

            1044   TCLQKVDEQPEDADNQRNVTRMGSQPSDPSTTVHVPVTLTGPPGEATVVP   1093
                   ||||||:|||||||||||||||||||.||.|.||:|| |||| |||||||
            1046   TCLQKVEEQPEDADNQRNVTRMGSQPPDPNTIVHIPVMLTGPLGEATVVP   1095

            1094   SANTDLEGQAEGKKEAEADDVLRRGPRPIVPYSSMFCLSPTNLLRRFCHY   1143
                   |:|.|||:|||||||.|||||:|.||||||||||||||||||||||||
            1096   SGNVDLESQAEGKKEVEADDVMRSGPRPIVPYSSMFCLSPTNLLRRFC..   1143
```

Figure 12

1	MKDRTQELRT	AKDSDDDDDV	TVTVDRDRFM	DEFFEQVEEI	RGFIDKIAEN
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101	EQEEGLNRSS	ADLRIRKTQH	STLSRKFVEV	MSEYNATQSD	YRERCKGRIQ
151	RQLEITGRTT	TSEELEDMLE	SGNPAIFASG	IIMDSSISKQ	ALSEIETRHS
201	EIIKLENSIR	ELHDMFMDMA	MLVESQGEMI	DRIEYNVEHA	VDYVERAVSD
251	TKKAVKYQSK	ARRKKIMIII	CCVILGIIIA	STIGGIFG	

Figure 13

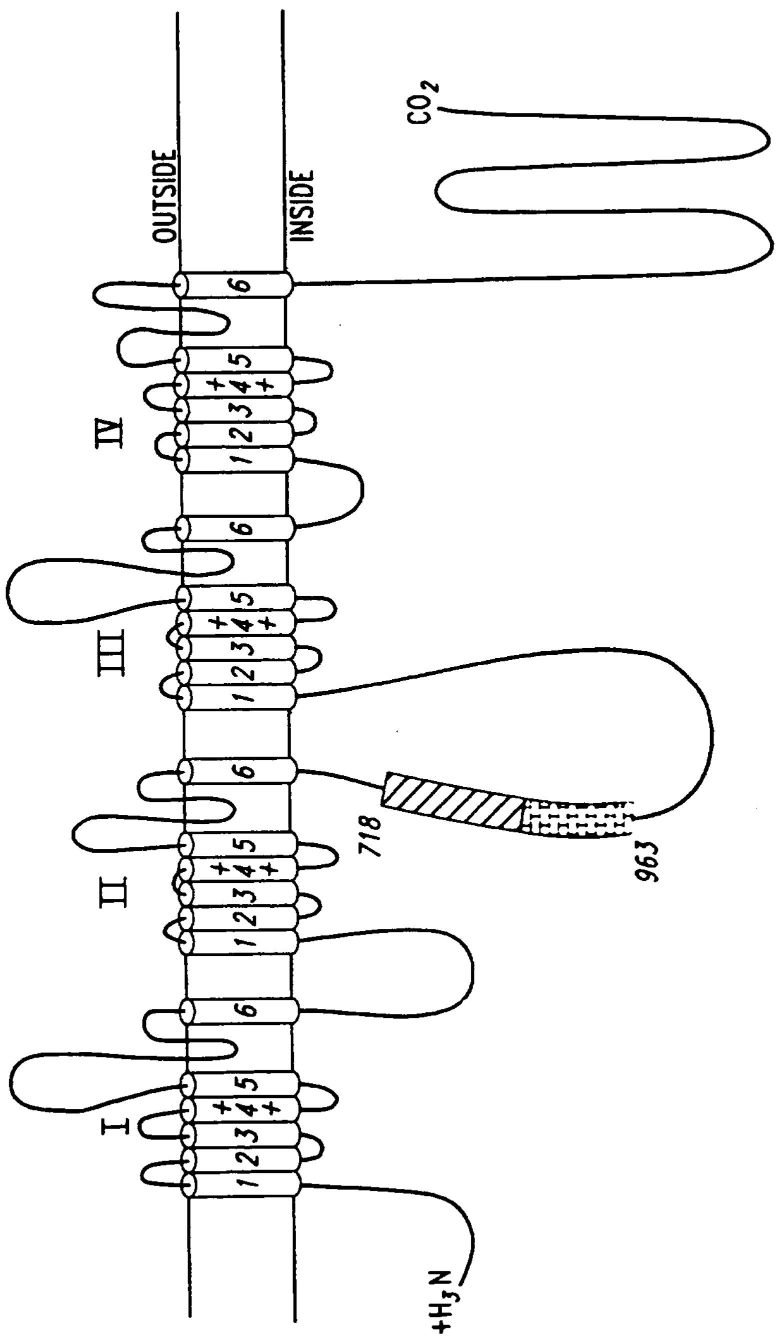

Figure 14

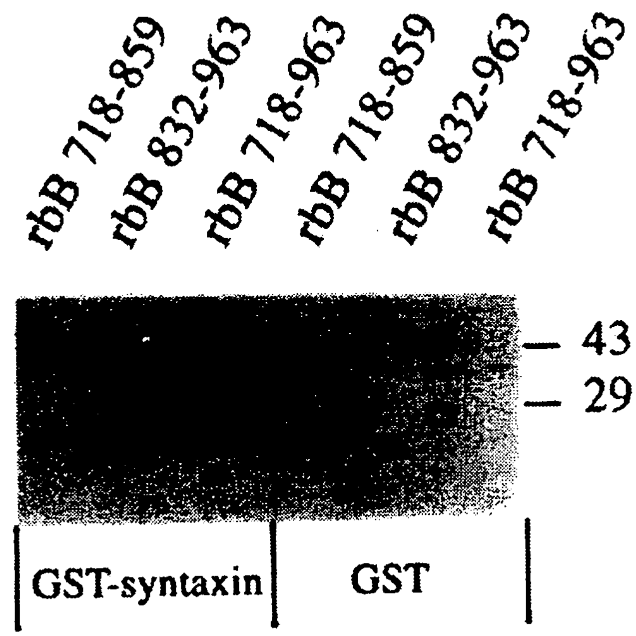
rbB 718-859
rbB 832-963
rbB 718-963
rbB 718-859
rbB 832-963
rbB 718-963
43
29
GST-syntaxin
GST

Figure 15

```
↓722
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↑724
                                                            ↓843
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LASREALYg--DaaERWpttYARpLRPDvKTHLDRPLVVDPQENRNNNTNKSRApEa---------
                                                           ↑844
                                            ↓895
RAEDFLRKQARhHDRARDPSahAaaGLDARRPWAGSQEAELSREGPYGRESDHQAREGgLEPPGF-
RAEDFLRKQARyHDRARDPSgsA--GLDARRPWAGSQEAELSREGPYGRESDHhAREGsLEqPGF-
-----LRqtARpresARDP--------DARRaWpsSpErapgREGPYGRESepQqREha--PPreh
                                         ↑869

--WEGEAERGKAGDPHRRHaHRQGvgGSggSRSGSPRTGtADGEPRRHRvHRRPGEdGPDDKAERR
--WEGEAERGKAGDPHRRHvHRQG--GSreSRSGSPRTG-ADGEhRRHRAHRRPGEEGPeDKAERR
vpWdadpERaKAGDapRRHtHRpv----------------AeGEPRRHRArRRPGdE-PDDrpERR

                                                       ↓1036
gRHREGSRPARsGEGEaEGPDGGggggERRRRHRHGPPpaYdpDARRDDRERR
aRHREGSRPARgGEGEgEGPDGG-----ERRRRHRHGaPAtYegDARReDkERR
pRpRdatRPARaadGE--GdDG------ERkRRHRHGPPAh-------DDRERR
                                                      ↑981
```
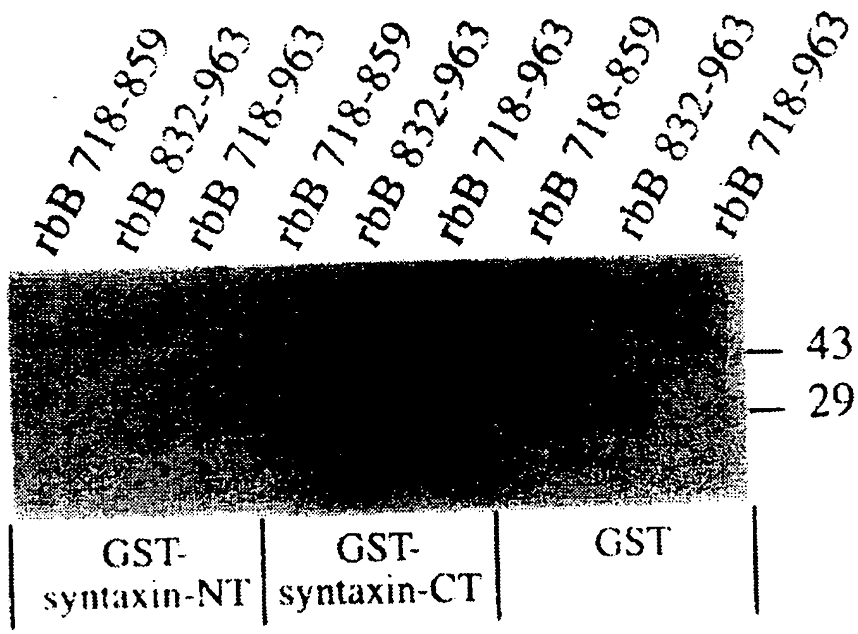

Figure 16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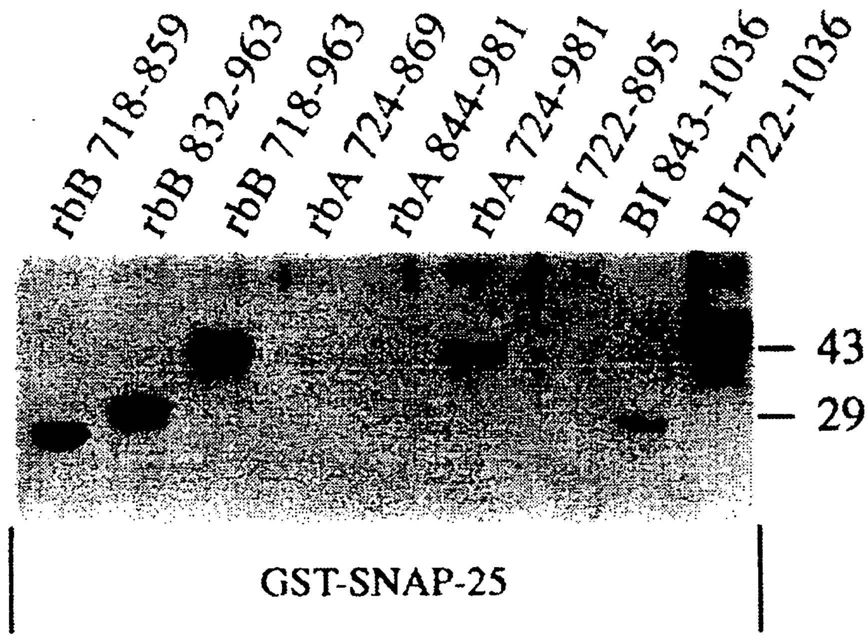
rbB 832-963
rbB 718-963
rbB 718-859
rbB 832-963
rbB 718-963
rbB 718-859
rbB 832-963
rbB 718-963
43
29
GST-syntaxin-NT
GST-syntaxin-CT
GST

Figure 17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rbB 718-963
rbA 724-869
rbA 844-981
rbA 724-981
BI 722-895
BI 843-1036
BI 722-1036
43
29
GST-SNAP-25

Figure 18